NADPH oxidase in the vasculature: expression, regulation and signalling pathways; role in normal cardiovascular physiology and its dysregulation in hypertension

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List of abbreviations

4EBP	Eukaryotic translation initiation factor 4E-binding protein
5-HT	5-hydroxytryptamine
16α-HOE	16α-hydroxyestrone
Ang II	Angiotensin II
ASK-1	Apoptosis signal-regulating kinase-1
BCL6	B-cell lymphoma 6
BH_4	Tetrahydrobiopterin
BK_{Ca}	Large conductance Ca^{2+} activated K^+ channel
BMPR2	Bone morphogenetic protein receptor-2
$[Ca^{2+}]_i$	Intracellular Ca^{2+} concentration
cADPR	Cyclic ADP-ribose
CAMK-II	Ca^{2+}-calmodulin activated kinase-II
CPI-17	C-kinase potentiated protein phosphatase-1 Inhibitor-17
CSK	c-Src kinase
DAG	Diacylglycerol
DCF	2,7-dichlorofluorescein
DHE	Dihydroethidium
DOCA salt rat	Deoxycorticosterone acetate salt rat
DPI	Diphenyleneiodonium
EBP50	Ezrin-radixin-moesin binding phosphoprotein 50
EDHF	Endothelium-derived hyperpolarising factor
EGFR	Epidermal growth factor receptor
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
FAK	Focal adhesion kinase
GPCR	G-protein coupled receptor
GRB2	Growth-factor receptor bound protein-2
GSH	Reduced glutathione
H_2O_2	Hydrogen peroxide
HETE	Hydroxyeicosatetraenoic acid
HIF-1α	Hypoxia-inducible factor-1α
HPV	Hypoxic pulmonary vasoconstriction
HSP27	Heat-shock protein 27
HSP90	Heat-shock protein 90
IGFR	Insulin-like growth factor receptor
IK_{Ca}	Intermediate conductance Ca^{2+}-activated K^+ channel
K_{IR}	Inward rectifier K^+ channel
ILK	Integrin-linked kinase
IP_3	Inositol trisphosphate
IP_3R	IP_3 receptor
JAK2	Janus kinase-2
K_V	Voltage-gated K^+ channel
LMW-PTP	Low molecular weight protein tyrosine phosphatase
LVGCC	L-type voltage-gated Ca^{2+} channels
MAP	Mean arterial pressure
MCT	Monocrotaline
MLC_{20}	Myosin-light chain-20
MLCK	Myosin light-chain kinase
MLCP	Myosin light-chain phosphatase

mTOR	Mammalian target of rapamycin
MYPT1	Myosin phosphatase targeting subunit-1
NAADP	Nicotinic acid adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO·	Nitric oxide
NOX	Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase
NRTK	Non-receptor tyrosine kinase
$ONOO^-$	Peroxynitrite
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PDI	Protein disulphide isomerase
PECAM-1	Platelet-endothelial cell adhesion molecule-1
PH	Pulmonary hypertension
PAH	Pulmonary arterial hypertension
PIP_2	Phosphatidyl inositol bisphosphate
PIP_3	Phosphatidyl inositol trisphosphate
PI3K	Phosphatidyl inositol-3 kinase
pKa	Dissociation constant for ionisation (of amino acid side-chains)
PKC	Protein kinase C
PKG	Protein kinase G
PLA_2	Phospholipases A_2
PLC	Phospholipases C
PLD	Phospholipase D
Poldip2	Polymerase delta interacting protein 2
PPARγ	Peroxisome proliferator-activated receptor gamma
PTP	Protein tyrosine phosphatase
PTP1B	Tyrosine protein phosphatase non-receptors type 1
PTPεM	Tyrosine protein phosphatase-εM
PTEN	Phosphatase and tensin homolog
PYK2	Protein tyrosine kinase 2β
RacGEF	Rac-specific guanine nucleotide exchange factor
RhoGEF	Rho-specific guanine nucleotide exchange factor
ROCK	Rho-activated kinase
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
RyR	Ryanodine sensitive channel
S1P	Sphingosine-1 phosphate
SERCA	Sarcoplasmic reticulum Ca^{2+} ATPase
SHP2	Src-homology region-2 domain-containing phosphatase-2
SHR	Spontaneously hypertensive rat
SK_{Ca}	Small conductance Ca^{2+}-activated K^+ channel
SOD	Superoxide dismutase
STAT	Signal transducer and activator of transcription
TGF-β	Transforming growth factor-β
TRPA1	Transient receptor potential ankyrin-1
TRPC1	Transient receptor potential canonical-1
TRPC6	Transient receptor potential canonical-6
TSP-1	Thrombospondin-1
TNF-α	Tumor necrosis factor-α
VEGFR	Vascular endothelial growth factor receptor

VSM	Vascular smooth muscle
VSMCs	Vascular smooth muscle cells
WKY	Wistar-Kyoto rat

Abstract

The last 20-25 years have seen an explosion of interest in the role of NADPH oxidase (NOX) in cardiovascular function and disease. In vascular smooth muscle and endothelium, NOX generates reactive oxygen species (ROS) that act as second messengers, contributing to the control of normal vascular function. NOX activity is altered in response to a variety of stimuli, including G-protein coupled receptor agonists, growth-factors, perfusion pressure, flow and hypoxia. NOX-derived ROS are involved in smooth muscle constriction, endothelium-dependent relaxation and smooth muscle growth, proliferation and migration, thus contributing to the fine-tuning of blood flow, arterial wall thickness and vascular resistance. Through reversible oxidative modification of target proteins, ROS regulate the activity of protein tyrosine phosphatases, kinases, G proteins, ion channels, cytoskeletal proteins and transcription factors. There is now considerable, but somewhat contradictory evidence that NOX contributes to the pathogenesis of hypertension through oxidative stress. Specific NOX isoforms have been implicated in endothelial dysfunction, hyper-contractility and vascular remodelling in various animal models of hypertension, pulmonary hypertension and pulmonary arterial hypertension, but also have potential protective effects, particularly NOX4. This review explores the multiplicity of NOX function in the healthy vasculature and the evidence for and against targeting NOX for antihypertensive therapy.

1. Introduction

Vascular smooth muscle (VSM) function is regulated by a highly complex network of intracellular signalling pathways incorporating cell surface receptors, G proteins, ion channels/transporters, kinases/phosphatases, cytoskeletal proteins/adaptors, transcription factors, all under the regulation of endocrine, neuronal, paracrine/autocrine, metabolic and mechanical stimuli. For these stimuli to contribute effectively to vascular function, they must be able to induce the production of second messenger molecules and these must be able to influence the function of specific cellular targets in a pre-determined spacio-temporal manner, and these effects must be readily reversible. Established second messenger molecules include cytosolic Ca^{2+}, cyclic nucleotides, phospholipids and more recently, reactive oxygen species (ROS). ROS can be defined as any electrophilic oxygen containing species, but in living cells, the primary species are superoxide ($O_2^{\cdot -}$) and its dismutation product hydrogen peroxide (H_2O_2), which are produced through one or two electron reduction of molecular oxygen, respectively. Electron transfer is catalysed by members of a large family of oxidoreductase enzymes. For many of these, including complex I and III of the mitochondrial electron transfer chain, uncoupled endothelial nitric oxide synthase (eNOS), xanthine oxidase, cyclooxygenases and lipoxygenases, ROS production is considered to be secondary to the principle function of the enzyme, at least under normal physiological conditions [1-7]. However, the main source of ROS in the vasculature (including in VSM and endothelium), is from the family of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX), for which their sole function is the production of superoxide and/or H_2O_2 [8-10]. In phagocytes the function of NOX is to generate an 'oxidative burst' the purpose of which is to kill and destroy engulfed pathogens. In non-phagocytic cells, including VSM and endothelium however, it is now known that NOX-derived ROS act as essential second messenger molecules contributing to the regulation of normal cellular function and their production is tightly regulated. Only during 'oxidative stress', a key feature of cardiovascular disease, are ROS produced in excess and considered harmful. Of the seven known isoforms of NOX, four are expressed in VSM and endothelium, namely NOX1, 2, 4, and 5.

Experimental and theoretical analysis of the chemical properties of superoxide and H_2O_2 in aqueous or hydrophobic environments suggests that both of these ROS species can modify protein function though direct oxidation of the sulfhydryl side chains (-SH) of cysteine residues [11, 12], resulting in conformational changes that may enhance or inhibit protein activity. Single electron oxidation (sulfenylation; R-SOH) is readily reversible through the combined action of thioredoxins, glutathione peroxidases and reduced glutathione (GSH). Additional oxidation of –SOH (sulfinylation; R-SO_2H) is also reversible through the action of sulfiredoxin-1, but further oxidation of –SO_2H (sulfonylation; R-SO_3H) is considered irreversible [13-15]. Cysteine oxidation may also result in reversible formation of intra-molecular or inter-molecular disulphide bonds that stabilize a particular tertiary or quaternary structure, again to either promote or inhibit function. Under normal conditions, cysteine oxidation is highly selective, being dependent on the dissociation constant (pKa) of the sulfhydryl group in question and therefore its propensity to donate electrons. In addition to direct oxidation by ROS, cysteine residues may be oxidised indirectly by oxidised peroxyredoxin (PRX) or nitrosylated (R-SNO) by the reactive nitrogen species, nitric oxide ($NO\cdot$) [16, 17]. Nitrosylation requires a prior single

electron oxidation (R-S·) and is reversible through the action of thioredoxin-1 and GSNO-reductase [17].

Thioredoxin may also catalyse trans-nitrosylation of cysteine by nitroso-glutathione (GSNO) [17], or cysteine may be directly glutathionylated by oxidised glutathione (R-SSG). Recent work has also suggested that ROS-dependent oxidation of methionine (R-SCHO) may also influence protein and vascular function and this is reversible through the action of methionine sulfoxide reductase [18, 19]. Cysteine, lysine and histidine residues may also be modified through the formation of Michael adducts with lipid peroxides (LOO-) such as 4-hydroxynonenal, generated by oxidation of poly-unsaturated fatty acids. Being lipids, this latter form of modification may be of particular importance for the oxidative regulation of membrane bound proteins, such as ion channels [20].

Superoxide spontaneously reacts with NO·, to form peroxynitrite ($ONOO^-$). $ONOO^-$ also modifies protein function by nitrating tyrosine residues (R-ONO), via reaction with CO_2 and formation of the intermediate nitrogen dioxide and carbonate anion radicals, NO_2· and $CO_3^{\cdot -}$ [17]. Nitration influences protein function by preventing or mimicking phosphorylation [21]. In the presence of free cytosolic Fe^{2+}, H_2O_2 is converted to the very powerful oxidant hydroxyl radical (OH•) which oxidises proteins indiscriminately, regardless of pKa, thus is unlikely to be of physiological relevance. Key targets for reversible oxidative modification by NOX-derived ROS in VSM function include a wide range of cell surface and intracellular proteins, including phosphatases, kinases and other cytosolic enzymes such as G-proteins and adapter proteins, ion channels and transporters and transcription factors.

This review will provide an update on expression, compartmentalisation, regulation and function of the different NOX isoforms expressed in the vasculature. It will detail the complex signalling networks both upstream of NOX activation and downstream of ROS production. It will report on how NOX activity is regulated by factors that influence VSM function, including vasoconstrictors, vasodilators, pressure/stretch, flow/shear-stress, mitogenic stimuli and hypoxia. Principle ROS effector proteins and downstream signalling pathways relevant to normal VSM function (contraction/relaxation) and the influence of endothelial factors on that function (NO·, endothelium-derived hyperpolarising factor, EDHF) will be examined. Also included is a summary of how the expression and function of specific NOX isoforms is altered in hypertension, with often conflicting evidence for and against their importance to the pathogenesis of the disease. The role of NOX4 is particularly contentious. Finally, there will be a discussion of NOX function in the pulmonary circulation, how it differs from systemic, its importance to the pulmonary vasoconstriction response to acute hypoxia and its potential role in pulmonary hypertension.

2. NADPH oxidase isoform expression and regulation in the vasculature

2.1. NOX isoforms. The first identified and best characterised of the NADPH oxidases is the membrane-spanning flavocytochrome b558, a heterodimer composed of $gp91^{phox}$ (now known as NOX2), and $p22^{phox}$. $gp91^{phox}$ is composed of six transmembrane α-helices connected by five loops, three of which are extracellular and the other two intracellular, while $p22^{phox}$ is composed of two transmembrane α-helices with cytosolic tails at both termini [22-24]. b558 generates superoxide by

the single electron reduction of two molecules of oxygen, the two electrons being transferred one at a time from a single molecule of NADPH at a dehydrogenase domain via FAD and two internally bound haem units [25]. This enzyme was identified as being responsible for the phagocytic respiratory burst in leucocytes [22], but soon after found to be expressed at considerably lower but functionally significant levels in both vascular smooth muscle cells (VSMCs) [26] and endothelium [27]. NOX2 requires specific stimuli for full activation, and collectively the intracellular loops of $gp91^{phox}$ and $p22^{phox}$ interact with cytosolic regulatory subunits $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$ following a sequence of signalling events resulting in their translocation from the cytosol where they reside as a non-active heterotrimer [28].

The essential steps in the activation of the phagocytic oxidase begin with activation of protein kinase C (PKC) and PKC-dependent phosphorylation of the cytosolic regulatory subunit $p47^{phox}$. This phosphorylation triggers cytosolic to membrane translocation of $p47^{phox}$, together with other cytosolic regulatory subunits $p67^{phox}$ and $p40^{phox}$, their association with $gp91^{phox}$ and $p22^{phox}$ and partial oxidase activation [29-31]. $p67^{phox}$ is thought to contribute to the dehydrogenation of NADPH and the transfer of e^- to FAD [30, 32-34]. Full oxidase activity also requires phosphatidyl inositol-3 kinase (PI3K) and the small G proteins Rac1 or Rac2. PI3K is required mainly because $p47^{phox}$ and $p40^{phox}$ also possesses phosphoinositide binding sites [35-37], while Rac1 binds the dehydrogenase domain of $gp91^{phox}$ and $p67^{phox}$ and is required for complete assembly and full activation of the oxidase [22, 30, 38]. $gp91^{phox}$ also appears to require direct phosphorylation by PKC during its activation [39]. As detailed below, vascular NOX2 is activated in a similar way, although the specifics of how PKC, PI3K and Rac are activated and subcellular distribution of subunits may vary.

There are now known to be seven isoforms of the catalytic subunit of NOX (NOX 1-5 and DUOX1 & 2), of which NOX 1, 2, 4 & 5 are expressed in the vasculature [23]. The relative abundance of NOX1 and NOX2 appears to vary depending on the cell type, size of vessel and vascular bed, as well as species and physiological/pathophysiological conditions [23, 26, 40-45]. Collectively, the literature suggests that both are expressed in both endothelium and VSMCs but NOX2 appears to be the dominant subtype in endothelium, while NOX1 predominates in VSMCs. NOX4 is ubiquitously expressed at high levels in both VSMCs and endothelium of humans and rodents [23, 45-53] and in one report, is 100-fold more abundant in endothelium than NOX2 [53]. NOX5 is highly expressed in the human vasculature, but curiously absent from rodents [54-57]. All other NOX/phox subunits expressed in phagocytes are also present in VSMCs and endothelium [23, 26, 58-60]. As in phagocytes, $p22^{phox}$ is essential for G-protein coupled receptor (GPCR)-induced NOX1/2 activity in the vasculature [61-63]. As with NOX2, $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and Rac also participate in the activation of NOX1 [64], but vascular cells and tissues expressing NOX1 also express homologues of $p47^{phox}$ and $p67^{phox}$, NOXO1 and NOXA1, respectively [23](discussed further in section 2.3).

Low levels of preassembled NOX1 or NOX2 [41, 60] together with active NOX4 [65] or NOX5 [66] probably contribute to basal ROS levels in unstimulated cells, and under these conditions, the rate limiting step for basal NOX-derived superoxide is probably the availability of cytosolic NADPH [67].

This 'basal' ROS undoubtedly contributes to normal resting cellular functions such as basal eNOS activity and resting smooth muscle tone, as well as making a contribution to ROS-dependent protein-folding in the endoplasmic reticulum [46, 51, 68-70]. However, NOX isoforms respond to external stimuli by generating brief bursts of ROS in the short term or sustained increases over longer periods. Over the short to medium terms (seconds to several minutes), enhanced NOX activity requires an initial stimulus which, through a series of signalling events, results in full activity of the enzyme. The exact nature of the stimulus, activation cascades and their effects depend largely on the NOX isoform being examined, its location in the cell and in which cell type it is expressed. As expected, acute stimuli for the production of ROS in VSM and/or endothelium include GPCR, flow/shear-stress, pressure/stretch and hypoxia, but also some growth factor and cytokine receptors (Table 1).

2.2. NOX1 and NOX2 'Canonical' activation pathway. GPCR agonists are the most frequently studied short term stimuli for NOX1 and NOX2 activity in the vasculature. The most extensively studied of the vascular GPCR agonists for NOX1 or NOX2 is angiotensin II (Ang II), acting via the type 1 angiotensin receptor (AT1R) to induce vasoconstriction, gene expression, growth, proliferation and migration [26, 40, 43, 45, 55, 71-108], but there are many others known to activate NOX in VSMC or endothelium through similar if not identical signalling pathways. These include endothelin-1 (ET-1) [55, 84, 109-114], ATP/purinergic agonists [64, 115-118], urotensin II [119-121], thrombin [62, 122-130], vasopressin [131], catecholamines (especially, alpha agonists) [131-137], 5-hydroxytryptamine (5-HT) [138], arachidonic acid and various eicosanoids, including prostaglandins, hydroxyeicosatetraenoic acids (HETEs) [6, 58, 139-146] and sphingolipids [147-150]. Here, common pathways for the activation of NOX in both VSM and endothelium are described. Additional activation by mechanical forces in the vasculature, most notably pressure/stretch in VSM and flow/shear-stress in endothelium, are discussed later.

The multiple steps required to be taken to achieve full and complete activation of NOX1 or NOX2 in vascular tissue are similar to those that apply to the phagocytic oxidase in leukocytes and, as illustrated in Figure 1, can be divided into two phases: (1) a first phase, corresponding to an initial rapid, often transient elevation in ROS production, and (2) a second phase, corresponding to a sustained longer lasting increase in ROS production. The most important step in the first phase is the phosphorylation and translocation of $p47^{phox}$ to the membrane, together with recruitment of $p67^{phox}$ and $p40^{phox}$ and their association with NOX1/2 and $p22^{phox}$. For this to occur, the initial stimulus must be capable of activating phospholipases C, D or A_2 (PLC, PLD, PLA_2) and the kinases PKC and/or Src, which then phosphorylate $p47^{phox}$. The requisite step in the second phase is the recruitment of Rac to the partly assembled enzyme. Activation of Rac and complete assembly of the oxidase appears to require further ROS-dependent activation of Src and activation of PI3K, often via transactivation of a receptor tyrosine kinase (RTK), usually epidermal growth factor receptor (EGFR). Each of these key steps are outlined below.

Phospholipase-dependent activation of PKC is an important first step in assembly of vascular NOX1 or NOX2 by GPCR agonists acting via Gq, although which phospholipase and which lipid products are considered most important for this activation appears to vary. Many GPCR expressed by VSMCs are

coupled to Gq and the activation of PLC, which generates inositol trisphosphate (IP_3) and diacylglycerol (DAG). Conventional PKC isozymes are activated by Ca^{2+} released from IP_3-sensitive stores and both conventional and novel isozymes are activated by DAG. Despite this, relatively few studies have directly shown NOX activation to be PLC-dependent in the vasculature. These include NOX2-dependent ROS production induced by Ang II in renal preglomerular VSMCs [133] and NOX1-dependent ROS production induced by sphingosylphosphorylcholine in mesenteric and pulmonary artery [148], sphingosine-1 phosphate (S1P) in rat aorta [147], advanced glycosylation end-products in retinal capillaries [151] or by thrombospondin-1 (TSP-1) in mouse aorta [152]. Alternatively, there is substantial evidence for a role of the PLD- or PLA_2-derived lipids phosphatidic acid, arachidonic acid or lysoposphatidylcholine, in activation of NOX by GPCR. PLD and PLA_2 are activated by G$\beta\gamma$ subunits [153, 154], while PLD activation in response to Ang II and other GPCR agonists also requires the small GTPase ARF6 [90, 108, 155]. In response to Ang II in VMSC, these lipid metabolites are likely to be activating PKC, since as outlined below, PKC-dependent serine phosphorylation of $p47^{phox}$ is a prerequisite for $p47^{phox}$ translocation to the membrane and its association with NOX1/2 and $p22^{phox}$, although which PKC isozyme is specifically shown to be PLD or PLA_2-dependent in this context is rarely specified [79, 80, 100, 156, 157]. Various stimuli In endothelium, including thrombin, low density lipoprotein and tumor necrosis factor-α (TNF-α) appear to couple PLA_2–derived arachidonic acid metabolites to NOX activity [124, 158-160], and two of these stimuli are likely to involve PKC as the target of those metabolites [158, 159].

There is strong evidence supporting a necessary role for PKC in NOX activation in VSM of multiple vascular beds and in some endothelial preparations, as demonstrated by the blocking effect of PKC inhibitors on NOX activity and ROS production in response to various stimuli including direct PKC activation by phorbol esters [67, 140, 161, 162]. These include Ang II in mouse and human coronary, human subcutaneous artery, rat aortic VSMCs and mouse renal preglomerular arterioles [77, 80, 100, 133, 152, 163], TSP-1 in mouse aorta [152], TNF-α or platelet-derived growth factor (PDGF) in human pulmonary endothelium and rat aortic VSMCs [158, 163], U46619 or sphingosylphosphorylcholine in bovine coronary, rat mesenteric or bovine aortic endothelium [6, 58, 140, 148] and direct PKC activators in rat or bovine aorta or bovine coronary artery [140, 156, 164]. Hypoxia is also capable of activating NOX via PKC [165] and is almost certainly doing so via upstream stimulation of mitochondrial ROS production (section 7.2), since PKC is also ROS-sensitive (section 3.3). Multiple studies have used $p47^{phox-/-}$ mice, siRNA or inhibitory peptides that target $p47^{phox}$ interaction with $p22^{phox}$, $gp91^{phox}$ and/or NOX1 at the membrane to show the dependence of the initial stimulated ROS production on $p47^{phox}$ in the vasculature [72, 93, 97, 104, 118, 132, 140, 144, 148, 152, 158, 165-168]. As in phagocytes, several studies have confirmed that the main role of PKC in vascular NOX activation is to serine phosphorylate $p47^{phox}$, triggering translocation of $p47^{phox}$ and $p67^{phox}$ to the membrane and their association with NOX and $p22^{phox}$ [6, 26, 58, 97, 118, 152, 158, 162, 169]. In addition, some studies have implicated specific PKC isozymes in vascular NOX activation, namely PKC-α [162], PKC-β [163, 170, 171], PKC-δ [169], PKC-ε [148, 165] or PKC-ζ [6, 108, 158]. There is also evidence for a role for phosphorylation of $p22^{phox}$, $p67^{phox}$ and $p40^{phox}$ by a range of serine/threonine kinases in non-vascular

cells [22, 30, 172], much of which may also be relevant in the vasculature but is yet to be demonstrated.

Interestingly, NOX activity in VSMCs and endothelium, unlike that in leukocytes, also appears to be dependent on tyrosine kinase activity, particularly the non-receptor tyrosine kinase Src, or other closely related kinases. Src is strongly involved in both pressor and vascular remodelling responses to Ang II and a range of other stimuli [101] and is strongly associated with ROS signalling in the vasculature eg. [173, 174] and reviewed in [175-177]. Multiple studies have used Src inhibitors, siRNA or $Src^{-/-}$ mouse to show the necessity of Src activity for either NOX1 or NOX2 activation in various vascular preparations, including bovine coronary artery VSMCs [140], rat or mouse mesenteric and pulmonary arteries [101, 148], renal preglomerular VSMCs [133], rat or mouse aortic VSMCs [77, 101], human coronary or subcutaneous arteries [82, 178] or human pulmonary endothelium [179]. Like PKC, activated Src can trigger $p47^{phox}$ translocation and initial NOX1/2 activation by direct tyrosine phosphorylation of $p47^{phox}$ [82, 140, 144, 179] or indirectly via phosphorylation and activation of PKC [173] which also phosphorylates $p47^{phox}$, as described above. Interestingly, Src may also be activated by PKC-dependent phosphorylation [133, 140, 180, 181], resulting in positive feedback between the two kinases. This association between the two kinases is potentially initiated by basal ROS since both kinases are ROS sensitive [176, 177] (sections 3.2 and 3.3). A number of additional pathways have been suggested for the activation of Src by GPCR, including direct activation by $G\beta\gamma$ subunits [182, 183]. Translocation of cytosolic subunits and NOX activation may also involve a degree of actin cytoskeleton re-organisation, via Src and/or PKC-dependent phosphorylation of actin-associated proteins. One study has suggested that before PKC-δ can phosphorylate and trigger the translocation of $p47^{phox}$ to the plasma membrane it must first phosphorylate smooth muscle-22α thus freeing itself from the actin cytoskeleton [169]. Others show in human VSMCs and endothelium that Src phosphorylates cortactin, facilitating phosphorylation of $p47^{phox}$ and its translocation to the membrane [85, 184, 185]. A third study has also suggested that translocation of $p47^{phox}$ and activation of vascular NOX1 is facilitated by binding of $p47^{phox}$ to ezrin-radixin-moesin binding phosphoprotein 50 [89].

Once the holoenzyme is assembled at the plasma membrane, full activation of vascular NOX1 or NOX2 also requires association of the catalytic subunit and $p67^{phox}$ with activated Rac1 or Rac2, as described above for the phagocytic oxidase. This has been shown for basal ROS and ROS production induced by GPCR agonists and growth factors in mouse, rodent and human VSMCs and bovine endothelium [62, 71, 77, 90, 144, 178, 186, 187]. Being a small G-protein, Rac requires activation by Rac-specific guanine nucleotide exchange factors (RacGEFs). RacGEFs in turn require phospholipid binding and/or tyrosine phosphorylation for their activation. RacGEF tyrosine phosphorylation is Src-dependent, either direct or via intermediary activation of other non-receptor tyrosine kinases focal adhesion kinase (FAK), protein tyrosine kinase 2β (PYK2) or Janus kinase-2 (JAK2) [188-192]. In response to GPCR stimulation, Src also indirectly contributes to activation of the lipid kinase PI3K via phosphorylation and transactivation of growth factor receptors (particularly EGFR) [77, 125, 130, 145, 149, 193-195]. Subsequently, PI3K-derived phosphatidyl inositol bisphosphate (PIP_2) and/or phosphatidyl inositol

trisphosphate (PIP_3) promote translocation and activation of Rac1 and complete NOX complex assembly at the membrane [77, 96, 196]. PIP_3 binds and activates multiple RacGEFs [197-199], while in neutrophils, but not yet shown in VSM or endothelium, PIP_2 and PIP_3 bind and provoke conformational changes in $p47^{phox}$ and $p40^{phox}$ thus promoting/stabilising NOX2 assembly [36, 37]. PIP_2 and PIP_3 production is also supported by concomitant inhibition of the lipid phosphatase PTEN (phosphatase and tensin homolog) by the same stimuli that activate PI3K [142], perhaps by the initial NOX-derived ROS [196, 200] (see also section 3.1).

Interestingly, a number of studies have shown that growth factor receptor transactivation and PI3K activation in vascular cells is also NOX-dependent [62, 149, 150]. A popular explanation for this is that partial activation of NOX generates sufficient ROS to further activate Src, promoting docking to and transactivation of the growth factor receptor and that the NOX/Src/EGFR/PI3K/Rac/NOX signalling axis constitutes multiple nested positive feedback loops between NOX1/2-derived ROS and ROS dependent signalling (particularly Src and PKC) [42, 77, 140]. Indeed, exogenously applied H_2O_2 stimulates NOX activity in lysed VSMC membrane fractions [165]. It has been suggested that this feedback loop also forms the basis of the late sustained phase of ROS production which persists for at least 30 min after the initial GPCR-induced stimulus in VSMCs, following an EGFR-independent transient rapid phase [77]. Finally, GPCR-induced EGFR transactivation in VSMCs may also occur through direct oxidative activation of EGFR [200], ROS-dependent matrix metalloprotease activation, and epidermal growth factor shedding [130, 136, 201] or via oxidative inhibition of the tyrosine phosphatase Src-homology region-2 domain-containing phosphatase-2 (SHP2) [121, 200]. These are all parallel pathways which can probably operate independently of Src. Of relevance to downstream signalling, activated growth factor receptors also act as docking sites for the assembly of growth and proliferation signalling complexes, including growth-factor receptor bound protein-2 (GRB2) and PI3K/AKT (section 6.3).

2.3. Other 'non-canonical' regulatory sub-units and NOX chaperones. NOX1 can form functional oxidase units at the membrane with NOXO1 instead of $p47^{phox}$ [202] and/or with NOXA1 instead of $p67^{phox}$ [126, 202, 203]. Unlike $p47^{phox}$, NOXO1 lacks the auto-inhibitory domain and PKC binding site, so does not require phosphorylation for its membrane targeting and association with NOX1/$p22^{phox}$, perhaps conferring a greater degree of constitutive activity to the oxidase than with $p47^{phox}$ [202], although NOX1 presumably still requires PI3K and Rac1 for full activity. Alternatively, NOX1 may also be activated by a combination of $p47^{phox}$ and NOXA1 [126, 203], indeed one study suggests that while human endothelium expresses $p67^{phox}$, it is completely absent from rodent aortic VSMCs and replaced by NOXA1 [203], and it has also been noted that NOXA1 expression in VSMC is lower than that of other cytoplasmic activators of NOX, thus introducing a possible rate limiting step for NOX1 activation, but suggesting regulation of NOXA1 expression may be a key way of altering ROS production in the long term [126]. Alternatively, in VSMCs, physical association between NOXA1 and NOX1 may be facilitated by direct PKC-β-mediated phosphorylation of NOX1 [163]. NOX2 may also be activated by NOXA1, but with lower affinity/efficacy than by $p67^{phox}$ [204]. NOX1/2 expression and activity are also positively regulated by protein disulphide isomerase (PDI) and heat-shock protein 90 (HSP90) in both

endothelium and VSM [205-207]. PDI forms a ROS-induced intermolecular disulphide bond with $p47^{phox}$ which is apparently necessary for NOX1 enzyme complex stability [208].

2.4. Regulation of NOX4 in the vasculature. NOX4 is expressed in both endothelium and VSM and according to some reports, is the most highly expressed NOX in both cell types [209, 210]. Like NOX1 and NOX2, NOX4 also possesses a dehydrogenase domain that requires FAD for the transfer of electrons to O_2 [211], but it differs in that it usually makes H_2O_2 instead of superoxide, presumably by the direct transfer of two electrons to each O_2 molecule rather than one to each of two O_2 molecules [45, 212-215], but may instead make superoxide in some intracellular compartments [212, 216]. Despite some reports that NOX4 preferentially uses NADH over NADPH as an electron donor [216, 217], other studies in isolated membrane preparations demonstrated a similar selectivity for NADPH as for other NOX isoforms [212]. Like NOX1 and NOX2, the catalytic subunit of NOX4 associates with $p22^{phox}$ and this binding is necessary for catalytic activity [213, 214, 216, 218, 219], but unlike NOX1 or NOX2, it does not require $p47^{phox}$, $p67^{phox}$, NOX01, NOXA1, $p40^{phox}$ or HSP90 for its activation [205, 213], suggesting that it exhibits a high degree of constitutive activity and makes a major contribution to basal ROS production in the vasculature, particularly basal H_2O_2 [45, 46, 65, 211-213, 215, 216, 220].

Detailed characterisation of signalling pathways for the acute activation of NOX4 is scarce, but there is certainly sufficient evidence to support the suggestion that it responds acutely to flow/shear-stress in endothelium [52] (section 5.1), GPCR and growth factor stimuli in endothelium [215, 221-223], and various GPCR agonists in VSM [45, 131, 224-226] (Table 2). Some studies suggest that NOX4 activity is supported by arachidonic acid [210, 224] the production of which is stimulated by some GPCR, but they disagree over whether NOX4 activation by this lipid is also dependent on Rac [223, 224, 227], as is the case with NOX1 and NOX2, or is entirely independent of it [210, 213, 222]. There is also disagreement over whether or not NOX4 responds to activators of PKC and therefore whether it requires serine/threonine phosphorylation for full activity [210, 216]. There is as yet no evidence that NOX4 activity is regulated by tyrosine phosphorylation. The only clearly defined cytosolic regulatory protein for NOX4 is polymerase delta interacting protein 2 (Poldip2), which is necessary for NOX4-mediated H_2O_2 production in VSMC [226, 228-230] and is a known actin and integrin-associated protein [49]. These relatively simple interactions are summarised in Figure 2. NOX4 is also expressed in subcellular organelles, such as the sarcoplasmic reticulum, but whether it can respond to acute stimuli at this location is unknown (see below).

2.5. Regulation of NOX5 in the vasculature. NOX5 is similar to other NOX isoforms in that it has six transmembrane domains, two hemes and a dehydrogenase domain that binds FAD and NADPH, but it is fully functional as an oxidase in the absence of both $p22^{phox}$ and any cytosolic regulatory subunits [231, 232], except perhaps for HSP90 which appears to be essential for the long-term stability of the enzyme complex [205, 233], perhaps by antagonising the enzymatic instability induced by caveolin in endothelial caveolae [234]. The primary site of acute regulation of NOX5 is an N-terminal cytosolic domain containing four EF hands that bind free Ca^{2+}, as well as Ca^{2+}-calmodulin binding domains, that collectively relieve oxidase auto-inhibition [235, 236]. As a result, NOX5-derived ROS production is

enhanced by stimuli that raise intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in endothelium and VSM, such as thrombin, Ang II, ET-1, U46619 and bradykinin, acting both via voltage-dependent extracellular Ca^{2+} influx and sarcoplasmic reticulum Ca^{2+}-induced Ca^{2+} release [55, 66, 232, 237]. NOX5 is therefore essentially constitutively active but with a 'basal' activity that is dependent on $[Ca^{2+}]_i$ [66]. However, in addition to this basal activity, the sensitivity of vascular NOX5 to increases in $[Ca^{2+}]_i$ may also be enhanced via phosphorylation by kinases such as PKC-α [238, 239], Ca^{2+}-calmodulin activated kinase-II (CAMK-II) [240], ERK-MAP kinase [241] or c-Abl [242], but inhibited by the Ca^{2+}-independent PKC-ε [239]. These various routes for the activation of NOX5 are illustrated in Figure 2. Since H_2O_2 elevates $[Ca^{2+}]_i$ (sections 4.2 and 4.3), NOX5 activation may also contribute to a positive feedback loop between multiple NOX isoforms [242], although this has yet to be demonstrated in vascular cells.

2.6. Compartmentalisation of NOX isoforms and ROS metabolism. Since ROS are generally highly reactive species, it has been suggested that for them to operate as effective selective second messenger molecules, the site of ROS production should be localized near to or in the same sub-cellular compartments as the target proteins or even be in direct contact with those target proteins in the same compartment. There is a growing body of evidence to support this [15, 48, 243-246]. As described above, all NOX isoforms expressed in the vasculature are located in the plasma membrane, but most are likely concentrated in or around specific structures on the cell surface. In VSM, plasma membrane NOX4 preferentially localises with vinculin and clustered integrins [48, 234], where presumably Poldip2 couples NOX4-derived ROS to activation of FAK, integrin-linked kinase (ILK) and RhoA at focal adhesions [49, 226, 229, 230, 247]. This may suggest a possible role for NOX4 in pressure/stretch-induced myogenic contraction (section 4.6) and VSM migration and proliferation (section 6.3). In endothelium, NOX1 and 2 are found in caveolae where they will associate with and be activated by the 'mechanosome' of the endothelial flow/shear-stress sensor. NOX5 is also located in caveolae, but is not known to be activated by flow. NOX4 is sensitive to flow but unlike NOX1, 2 and 5, it is not located in the caveolae of endothelial cells and therefore caveolin cannot influence its activity in the short term, but it does negatively regulate its expression [234]. In caveolae, NOX1, 2 and 5 are maintained in a resting state by caveolin-1 through disruption of the enzyme complex until this is relieved by HSP90 [205, 233]. NOX4 is presumably located elsewhere on the surface of endothelial cells, but to which structures it binds is unknown. The role of endothelial cell surface NOXs in sensing flow is discussed in more depth later in this review (section 5.1).

Due to the orientation of NOX catalytic subunits in the plasma membrane, they will be producing primarily extracellular ROS. However, NOX4-derived H_2O_2 is free to diffuse through the plasma membrane to have intracellular actions, and this may be facilitated by cell surface aquaporins, widely expressed in the cardiovascular system [248-250] while for the negatively charged superoxide generated by NOX1, 2 or 5 to also have intracellular actions, transport must be facilitated by plasma membrane anion channels, including the chloride channel ClC-3 [251] and the volume sensitive channel LRRC8A [252], or it must first be dismutated to H_2O_2 by extracellular superoxide dismutase (SOD) [15, 253]. Alternatively, it is now fairly well established that functional NOX enzymes are also

located within various subcellular organelles, including the endosomes, sarcoplasmic reticulum, nucleus and mitochondria.

It is well known that growth factor receptors, cytokine receptors and GPCR are endocytosed upon ligand binding or following transactivation in the case of RTKs, either to be recycled back to the plasma membrane or sent to lysosomes for degradation. However, it is also now known that key signalling events occur in the endosome following receptor activation [243, 252]. It has been suggested that this includes activation of NOX1 in VSM [130, 252, 254, 255], and for activation of NOX2 in endothelium [256, 257]. This appears to occur following endocytosis of the activated NOX enzyme together with the activating receptor. For NOX1 and NOX2, this may be facilitated by $p40^{phox}$-dependent interaction with phosphoinositides in the endosomal membrane [36] or NOX-dependent phosphorylation of dynamin or caveolin-1 [252]. NOX4 and NOX5 are also found in endosomes and therefore may also contribute to endosomal RTK or GPCR signalling [242, 258]. Also endocytosed are several associated activator and effector proteins, such as Src, Rac and tyrosine phosphatases [245, 255, 258, 259]. Endosomes also contain SOD1, so superoxide can be dismutated to H_2O_2, enabling both NOX-derived ROS species to contribute to cell signalling in the endosome as a 'redoxosome' [15, 245, 246, 254, 257, 258, 260]. Alternatively, there is evidence that endosomal superoxide may be released into the cytosol through volume-sensitive anion channels (LRRC8) [252] or ClC-3 [254, 255, 261] and of course, H_2O_2 is also free to diffuse into the cytosol.

NOX4 is also co-expressed with $p22^{phox}$ in the sarcoplasmic reticulum of endothelium and VSM [46, 51, 53, 219, 225] and probably generates ROS within the lumen of the organelle [219]. The H_2O_2 produced here is also free to diffuse out or exit the compartment via aquaporins [15], but specific functions for NOX-derived ROS within the endoplasmic reticulum have been considered. In particular, it may contribute to the oxidising environment required for normal disulphide-dependent protein folding [15, 68] or contribute to the oxidative modification of Ca^{2+} release channel function (section 4.3). NOX5 is also expressed in the endoplasmic reticulum of endothelium [232], but its functions there are unknown. A functional splice variant of NOX4 lacking transmembrane domains but which still associates with $p22^{phox}$ has been detected in the nucleus of VSMCs where it may directly contribute to oxidative regulation of gene expression [216, 262, 263]. NOX4 is also reportedly expressed in the mitochondria of cardiac myocytes [264, 265], but whether it is also expressed there in VSM or endothelium and what its function may be is unclear. The mitochondria also generates superoxide through leakage of electrons from the inner membrane electron transport chain [1, 2] and this is of particular functional importance in vascular responses to hypoxia, where it may be coupled to plasma membrane or endosomal NOX activity (sections 7.2 and 7.3). Movement of ROS from within mitochondria and sarcoplasmic reticulum into the cytosol is also facilitated by aquaporins and ClC-3 (or equivalent) [15].

The multiple feedforward and positive feedback loops between ROS and upstream signalling pathways derived from NOX1 or NOX2 will ensure the response to a stimulus is rapid and amplifiable, but for this to be of any use as a controllable cell signal and to contribute to the highly compartmentalised

nature of the response there must also be an 'off-switch'. For feedback involving Src and PKC it has been suggested that this may arise out of the fact that as well as being activated by ROS, the two kinases are also inactivated by ROS, but the latter perhaps only at higher concentrations [176, 244, 266]. Apart from this, cells possess powerful antioxidant enzyme systems capable of limiting the accumulation and diffusion of any ROS generated and to some extent these too are compartmentalised. If superoxide does not react with target proteins or with NO· to produce $ONOO^-$, then it will eventually be dismutated to H_2O_2. Superoxide dismutase is present in the cytosol and endosomes (SOD1) [260], mitochondria (SOD1, MnSOD/SOD2) and extracellular space (EC-SOD) [15]. While H_2O_2 is more freely diffusible and less reactive than superoxide, its ultimate complete reduction to H_2O is catalysed by cytosolic catalase, or by peroxiredoxins or glutathione peroxidases located in the cytosol, mitochondria and endoplasmic reticulum [15]. The action of glutathione peroxidase is supported by the main cellular REDOX buffer, reduced glutathione (GSH). Oxidised glutathione (GSSG) is re-reduced to GSH by glutathione reductase utilising NADPH, which is itself generated in the pentose phosphate pathway, the ultimate source of cellular reducing equivalents. The compartmentalisation and metabolism of ROS are illustrated in Figure 3.

3. Proximal targets for NOX-derived ROS in the vasculature.

A wide range of signalling proteins involved in vascular smooth muscle function have been identified as being sensitive to ROS and often particularly sensitive to NOX-derived ROS. For many of these, the target protein is probably not directly sensitive to cysteine oxidation but is responding to ROS-induced modulation of an upstream 'proximal ROS target'. Downstream effector pathways include multiple protein kinases, ion channels, guanine nucleotide exchange factors for small G-proteins of the Rho family, cytoskeletal proteins and transcription factors. Their roles in NOX-dependent vascular function will be detailed in subsequent sections. This section will focus on essential proximal ROS sensors, protein tyrosine phosphatases (PTPs) and non-receptor tyrosine kinases (NRTKs), as well as PKC and CAMK-II.

Tyrosine phosphorylation has long been known as an essential early signalling event in response to growth factors and cytokines, since growth factor receptors are themselves tyrosine kinases and cytokine receptors directly activate non-receptor tyrosine kinases as an essential early step in their signalling cascades [21, 191, 267, 268], but more recently it has also been shown that vascular smooth muscle pro-contractile stimuli, GPCR agonists in general, stretch in many vascular beds and hypoxia in the pulmonary circulation, are also associated with enhanced total cellular tyrosine phosphorylation in the vasculature [21, 103, 269-273] and reviewed in: [176, 177]. Enhancements in cellular tyrosine phosphorylation induced by either growth or contractile stimuli in VSM are inhibited by antioxidants [267, 274, 275] or SOD and/or catalase [267, 276] and altered by inhibition/over-expression of NOX [103, 259, 274, 277, 278]. Similarly, exogenous ROS cause an increase in tyrosine phosphorylation in vascular tissues [103, 174, 191, 273, 279-283]. Net tyrosine phosphorylation and thus relative activity of the phosphorylated target proteins will always be determined by the relative opposing activities of tyrosine phosphatases and kinases [284]. For example, tyrosine phosphatase inhibitors generally induce constriction in vascular smooth muscle, whereas tyrosine kinase inhibitors induce relaxation

[82, 273, 279, 283]. As discussed in previous sections, all the stimuli mentioned above induce NOX-derived ROS production in the vascular wall, therefore the observed NOX-dependent effects of ROS on net tyrosine phosphorylation are most likely occurring via the combined effects of ROS-induced PTP inhibition and ROS-induced NRTK activation. As discussed below, there is a direct link between the induced NOX activity and the stimulation of downstream contractile and mitogenic effector pathways.

3.1. Protein tyrosine phosphatases. Tyrosine phosphatases, dual-specific phosphatases and the lipid phosphatase PTEN share a unique signature motif $HC(X)_5R(S/T)$ containing a nucleophilic cysteine which confers an unusually low pKa for ionization of 4-5 compared to the typical pKa of nonreactive cysteines of 8.5 in most other proteins [285]. Oxidation usually results in the formation of an intramolecular disulphide or sulfenamide bond which inhibits phosphatase activity by preventing docking to protein targets and relief of auto-inhibition. Like most serine threonine phosphatases, reduced PTPs (under low ROS conditions) do not require external stimuli for their activation and will be constitutively active when bound to target phospho-proteins, but will be progressively inactivated when cells are stimulated to increase ROS production, [196, 244, 286-290]. In some cases, as with PTEN, the phosphatase is protected from oxidation by PRX, but oxidation of PRX results in disulphide formation and inactivation of the phosphatase [16]. This oxidative inhibition is readily reversible through the action of glutathione peroxidase or thioredoxin [268, 288, 290].

Key tyrosine phosphatases important in the control of VSM or endothelial function that are sensitive to NOX-derived ROS include SHP2 [121, 291], PTP1B (tyrosine protein phosphatase non-receptors type 1) [51, 276], protein tyrosine phosphatase-εM (PTPεM) and low molecular weight protein tyrosine phosphatase (LMW-PTP) [292, 293]. All of these are key negative regulators of growth factor receptors and the signalling complexes that are activated by them. SHP2 is particularly important in suppressing activity of EGFR and platelet-derived growth factor receptor (PDGFR), by dephosphorylating phospho-tyrosines essential for the docking and activation of PI3K and GRB2. This may occur in endosomes co-expressing growth factor receptors and NOX1 or NOX4 and contributes to the feedback between RTK transactivation, PI3K activity and NOX activation [258]. PTP1B negatively regulates vascular endothelial growth factor receptor (VEGFR) signalling in endothelium [276] and appears to co-localise with NOX4 and EGFR in the endoplasmic reticulum where oxidative inhibition of the phosphatase promotes EGFR trafficking [51]. There is also some evidence that dual-specific tyrosine/threonine phosphatases are ROS-sensitive. This class of phosphatase is responsible for the de-phosphorylation and inactivation of MAP kinases and the oxidative inactivation of MAP kinase phosphatase-1 contributes to MAP kinase activation in VSM [294]. As mentioned above, oxidative inhibition of PTEN contributes to NOX1/2 activation by supporting PI3K-derived PIP_2 and PIP_3 production [196], although this has yet to be directly demonstrated in vascular tissues. PIP_3 is also required for the activation of AKT, thus PTEN is a possible route through which ROS may promote VSM growth. Obviously, phosphatases will oppose the actions of kinases by dephosphorylating downstream target proteins, such as ion channels, guanine nucleotide exchange factors and other kinases, but PTPs also de-

phosphorylate tyrosine residues on kinases themselves and these phospho-tyrosines may be inhibitory or stimulatory of kinase activity. Indeed most tyrosine kinases possess both (See below).

3.2. Receptor and non-receptor tyrosine kinases. There are two major families of tyrosine kinase, the receptor tyrosine kinases (RTK) and the non-receptor tyrosine kinases (NRTK). A feature shared by all tyrosine kinases is that they require auto-phosphorylation for full kinase activity. Most growth factor receptors are RTKs, and auto-phosphorylation is triggered by agonist binding and receptor dimerization or by transactivation involving phosphorylation by another kinase, usually an NRTK such as Src, in response to other stimuli, usually GPCR. This generates phospho-tyrosine docking sites for multiple downstream signalling pathways, including PI3K, GRB2 and PLC-γ. There are multiple families of NRTK, including the Src family kinases (notably Src, Fyn & Yes in vascular tissues), FAK, the closely related PYK2 and Janus kinase (especially JAK2). These kinases are important for mediating growth factor and cytokine-induced gene expression and growth-related signalling but are also key regulators of smooth muscle electrical excitability, intracellular Ca^{2+} and actin cytoskeleton dynamics, and therefore contractility. All of the above have, in one way or another, been implicated in ROS-dependent signalling in the vasculature and are either upstream or downstream of NOX-dependent ROS production, and make a major contribution to the control of smooth muscle tone induced by GPCR agonists [133, 174, 176, 177, 272, 295, 296], stretch [194] and hypoxia [174, 271, 297] (section 7.2), or the control of VSM proliferation [95, 298-300] (section 6.3).

Src (the principle member of the Src-family kinases) is one of a handful of protein kinases known to be directly ROS-sensitive, although the majority of evidence for this stems from extensive work in non-vascular cells and tissues. Src possesses two key phospho-tyrosine residues, an inhibitory one on the C-terminal tail (Y527 in humans) and a stimulatory one on the kinase activation loop (Y418 in humans). When Y527 is phosphorylated by c-Src kinase (CSK), internal SH2 binding promotes folding of the kinase, hiding the active site, and disruption of this internal binding by de-phosphorylation of Y527, or PKC-directed serine phosphorylation of the SH2 domain, triggers auto-phosphorylation of Y418, fully activating the kinase [301, 302]. Src also contains several reactive cysteine residues, including one in the SH2 domain and one in the kinase domain, oxidation of which induces hyper-phosphorylation of Y418 [303], and/or intermolecular disulfide bridge formation that may prevent internal Y527/SH2 binding, thus prolonging kinase activity [304]. Oxidation of these two cysteine residues and Src activation occurs in response to NOX activation [305]. ROS may also module Src activity indirectly via oxidative inhibition of CSK and PTPs that de-phosphorylate Y418 (stimulatory of Src) or of PTPs that dephosphorylate Y527 (inhibitory of Src). The former may ensure a rapid response to and positive feedback to NOX activation, while the latter may contribute to a timely termination of the signal [301, 306-308]. In line with Src activity generally having a positive relationship with NOX1/2-derived ROS generation, there is evidence that exogenous ROS activate Src and that VSM contractile or proliferative stimuli induce ROS-dependent or NOX-dependent Src oxidation and auto-phosphorylation [125, 173, 174, 259, 303, 309-312], and stimuli that activate NOX-dependent contractile responses and contractions induced by exogenous ROS are both suppressed by inhibitors of Src family kinases [148, 174, 177, 311].

Several other NRTKs, including FAK, PYK2 and JAK2, are ROS sensitive in the vasculature, but unlike Src, are probably not directly ROS-sensitive. The likeliest route is downstream of Src, which phosphorylates these kinases, triggering stimulatory auto-phosphorylation [145, 309, 313-315]. Alternatively, it may occur downstream of oxidative inhibition of a PTP that de-phosphorylates their activation loop [189, 291], or phosphorylation by a ROS sensitive PKC [316]. Either way, they are activated by GPCR agonists and other contractile, migratory or mitogenic stimuli in a manner dependent on NOX and endogenous ROS production or by exogenous ROS [109, 110, 126, 189, 191, 229, 309, 317, 318]. A couple of these studies have implicated specific NOX isoforms in activation of NRTK: JAK2 is stimulated by PDGF via NOX5-derived ROS [317] while FAK is activated by PDGF at focal attachments in a NOX4/Poldip2 dependent manner [229] and subsequently phosphorylates multiple focal adhesion associated proteins [319]. As discussed earlier, transactivation of growth factor receptors such as EGFR, PDGFR and insulin-like growth factor receptor (IGFR) is generally considered to be indirectly NOX and ROS-sensitive because the NRTK (usually Src) that initially phosphorylates the receptor in response to NOX activation is itself ROS-sensitive, as discussed above [125, 173, 194, 309]. As far as can be determined, only one study also suggests that EGFR transactivation may also involve direct sulfenylation of the EGFR kinase domain [200].

3.3. Protein kinase C and Ca^{2+}-calmodulin-activated kinase II. PKC is a family of 12 cytosolic serine/threonine kinases and like Src, it appears to be both upstream of NOX activation and a directly ROS-sensitive NOX effector. Six PKC isozymes of three classes are expressed in vascular tissues: conventional (PKC-α, -β_1, -β_2), novel (PKC-δ and -ε) and atypical (PKC-ζ) [320, 321]. All PKC isozymes are inactive when maintained in a folded configuration due to a regulatory domain containing an auto-inhibitory pseudo-substrate, which prevents access of the catalytic domain to substrate proteins. Unfolding and release of the pseudo-substrate is normally induced by Ca^{2+} (α and β only), DAG (α, β δ and ε only), other lipid mediators (all isozymes) and by tyrosine phosphorylation (all isozymes). Src is a key NRTK activator of PKC; reviewed in: [173, 176, 321]. Essential to the release of the pseudo-substrate are multiple cysteine residues in the regulatory domain and these are also highly sensitive to intramolecular disulphide bridge formation [322]. Cysteine oxidation removes the requirement for Ca^{2+}, or lipid mediators, automatically triggering unfolding and activation of the kinase [176, 321]. There is also evidence that cysteine residues on the catalytic domain of PKC may also be sensitive to oxidation, but that this oxidation inhibits kinase activity [323]. However, this appears to require higher concentrations of ROS than oxidation of regulatory domain cysteine residues, so may contribute to the termination of the positive feedback between PKC and NOX once sufficient ROS have been generated [176, 323] (section 2.6). These biochemical studies were all performed in non-vascular systems, but are supported by studies in vascular tissues showing that PKC undergoes cytosolic to membrane translocation and is activated in response to exogenous ROS in VSM or endothelium [324-327] and that inhibition of specific PKC isozymes inhibits ROS-induced or ROS-dependent GPCR mediated contraction and associated signalling events in isolated arteries [148, 283, 325-327]. CAMK-II is another kinase that is potentially both upstream (specifically of NOX5) and downstream of ROS. This kinase is normally activated by Ca^{2+}-calmodulin, binding of which triggers autophosphorylation at

Thr287, but is also activated by oxidation of two methionine residues (Met281/282). This oxidative activation is maintained independently of Ca^{2+}, but has the same effect on kinase activity [19, 328].

4. Role of NOX in VSM contraction.

VSM constriction negatively influences tissue blood flow while positively influencing vascular resistance and perfusion pressure. Thus, in order to match blood flow to the metabolic demands of the tissue, prevent tissue damage due to over-perfusion, contribute to thermoregulation and inflammation, or to regulate total peripheral resistance and MAP, VSM tone is under the control of circulating hormones (eg Ang II), peripheral neurotransmitters (noradrenaline, acetylcholine), paracrine and autocrine factors (bradykinin, histamine etc) haemodynamic forces (pressure/stretch, flow/shear-stress) as well as products of metabolism and PO_2. Interestingly, NOX and the two principle ROS, superoxide and H_2O_2, contribute to both contraction and relaxation responses in the vasculature, depending on the vascular beds and the nature of the stimulus. This has in part been investigated by testing the effects of application of exogenous H_2O_2 and artificially generated intracellular superoxide with xanthine/xanthine oxidase or cell permeable quinones such as LY83583 or menadione [329, 330] (Table 3). Despite being considered somewhat of a blunt instrument due to the use of potentially supra-physiological concentrations and the loss of subcellular compartmentalisation [244], the results of such experiments are largely supported by pharmacological and genetic manipulation of specific NOX isoforms as well as the use of non-specific antioxidants and highly selective antioxidant enzymes (SOD and catalase). There are several effector pathways through which ROS influence VSM contraction and relaxation, acting on both the VSM and the endothelium. The simplest is by a shift in the balance between the availability of eNOS-derived NO· and NOX-derived superoxide in favour of superoxide and/or a change in the ability of SOD to remove superoxide. This results in more superoxide chemically reacting with and 'scavenging' NO·, generating the poor vasodilator $ONOO^-$, thus impairing endothelium-dependent relaxation and enhancing vascular tone [99, 102, 152, 331-333]. However, this mechanism by no means fully accounts for the observed effects of superoxide in the vasculature because inhibiting or ablating eNOS does not abolish the effect [137, 311, 334-336]. The alternatives are more complex. As discussed throughout this section and illustrated in Figures 4 and 5, ROS can induce phosphorylation of myosin-light chain-20 (MLC_{20}), a necessary step in actin-myosin cross-bridge cycling and contractile force generation, either by directly influencing VSM $[Ca^{2+}]_i$, which induces phosphorylation of MLC_{20} by myosin light-chain kinase (MLCK), or by inhibition of myosin light-chain phosphatase (MLCP). There is also some evidence for a role in MLC_{20}-independent effects on actin dynamics. In the following sections, evidence for the role of specific NOX isoforms in mediating contractile and relaxation stimuli will be discussed. Please note that many of the studies cited in the following sections have made use of pharmacological agents designed as specific inhibitors of NOX, such as apocynin, DPI and VAS2870, but which have subsequently been queried as non-specific flavoprotein inhibitors or even as general antioxidants rather than NOX inhibitors, particularly apocynin [337, 338]. Nevertheless, such studies are included here because they are by and large supported by parallel studies utilising specific NOX isoform/regulatory subunit siRNA, over-expression and knock-out/knock-in mouse models, as well as newer NOX inhibitors and antioxidant enzymes. Conclusions drawn from these studies are also corroborated by the parallel use of ROS measurement

and/or exogenous ROS, often in the same articles. Please see Tables 2 & 4 for an overview of the relevant literature in this regard.

4.1. Pro-contractile actions of exogenous ROS. In the absence of pre-constriction, superoxide directly constricts rat aorta [339] and rat pulmonary artery [311, 340], but not mesenteric or femoral artery [311]. In the absence of pre-constriction, H_2O_2 directly constricts mouse and rat aorta [131, 341-344], bovine coronary artery [194], canine cerebral artery [283], mouse carotid [342] and rat or rabbit pulmonary artery [146, 326, 340, 343, 343, 345-347], but may or may not directly constrict rat mesenteric artery [341, 341-343, 348, 348]. In various mammalian systemic arterial preparations pre-constricted with GPCR agonists or myogenic tone, both superoxide and H_2O_2 appear to exert mixed, concentration-dependent, contraction/relaxation effects [311, 325, 327, 334, 343, 349, 350], although the dominant effect of H_2O_2 tends to be relaxation (section 5). Superoxide consistently enhances GPCR-induced pre-constriction in pulmonary artery [174, 311, 351] (section 7.1.). Alternatively, in various mammalian systemic vascular preparations, both H_2O_2 and superoxide consistently enhance sub-maximal contraction induced by KCl [148, 341, 342, 344, 350-354], because KCl removes the influence of ROS on K^+ channels (as explained further in section 5.5). Note that in some studies, superoxide generating systems X/XO or LY83583 reportedly cause contraction that is largely suppressed by SOD or only partly suppressed by catalase, suggesting that the contractile effect of superoxide does not necessarily require conversion to H_2O_2 [311, 339, 340, 351]. It is also worth highlighting that vasoconstriction in various vascular beds induced by exogenous ROS is inhibited by non-specific tyrosine kinase inhibitors [283, 341, 346], specific blockers of Src family kinases [174, 311, 352], and PKC inhibitors [283, 325, 326], suggesting that these proximal kinases contribute to ROS-induced VSM contraction either as direct ROS-sensors or downstream of PTP inhibition. There is evidence supporting a role for NOX-derived ROS, as well as Src and/or PKC, in all three of the main VSM contraction effector pathways (an increase in $[Ca^{2+}]_i$, inhibition of MLCP and actin polymerisation).

4.2. NOX/ROS-dependent Ca^{2+} influx pathways. Smooth muscle contraction is primarily dependent on phosphorylation of MLC_{20} by the Ca^{2+}-calmodulin-dependent MLCK. Exogenous H_2O_2 and superoxide applied in the absence of pre-constrictor agents induce increases in $[Ca^{2+}]_i$, in aorta and cerebral or mesenteric arteries [131, 283, 325, 348], while in pulmonary artery, H_2O_2 does [326], but superoxide does not [311]. However, superoxide does enhance GPCR-induced Ca^{2+} responses in pulmonary artery [351]. A major source of this ROS-induced $[Ca^{2+}]_i$ response appears to be influx via L-type voltage-gated Ca^{2+} channels (LVGCC), particularly in systemic vessels. This is based partly on the inhibitory effects of specific LVGCC antagonists on exogenous ROS-induced $[Ca^{2+}]_i$ and contraction in aorta and cerebral, mesenteric and renal arteries [283, 325, 327, 344, 348, 354, 355] and the enhancement of whole-cell L-type Ca^{2+} currents in mesenteric artery VSMCs by superoxide [148] and in cerebral artery by H_2O_2 [105]. NOX-derived endogenous ROS also contribute to GPCR-induced $[Ca^{2+}]_i$ influx and contraction in part via enhanced opening of LVGCC. For example, in bovine pulmonary artery VSMCs, the U46619-induced $[Ca^{2+}]_i$ increase is partially sensitive to inhibition of either NOX1/2 or LVGCC [58], Ang II-induces apocynin or catalase-sensitive Ca^{2+} sparklets and contraction in rat

cerebral artery [105, 325] and sphingosylphosphorylcholine induces antioxidant-sensitive voltage-gated Ca^{2+} current in mesenteric artery VSMCs in association with $p47^{phox}$-dependent contraction [148]. In addition, mouse mesenteric artery expressing human NOX5 exhibit enhanced antioxidant-sensitive contraction induced by depolarisation with sub-maximal KCl [66]. It is not clear whether cell surface NOX4 regulates Ca^{2+} channel activity in VSM but it does activate transient receptor potential canonical-6 (TRPC6) in podocytes in response to Ang II stimulation [356].

There is conflicting evidence over the influence of ROS on LVGCC activity through direct cysteine oxidation or S-nitrosylation, some suggesting that it is stimulatory [357, 358] and others that it is inhibitory [359-362], perhaps depending on which region of the channel is targeted. Alternatively, ROS-dependent stimulation of LVGCC Ca^{2+} influx may be indirect, either via oxidative activation of protein kinases that activate the channel by phosphorylation, or via prior action on other membrane channels that depolarises the plasma membrane, thus opening LVGCC. VSM LVGCC open probability is increased by both serine/threonine and tyrosine phosphorylation, most probably PKC, CAMK-II and/or Src-mediated, because the pore-forming subunit of LVGCC ($Ca_V1.2$) is a phosphorylation target for all three kinases [325, 358, 363-365]. PKC-α contributes to Ang II-induced NOX-dependent induction of LVGCC Ca^{2+} sparklet activity and contraction in rat cerebral artery [325]. CAMK-II enhances H_2O_2 induced $Ca_V1.2$ current in ventricular myocytes [358]. While there is no direct evidence that Src phosphorylates $Ca_V1.2$ in response to ROS, it is a reasonable assumption, considering it is itself a key ROS target in VSM. Conceivably, ROS could depolarise VSM by either inhibiting K^+ channel activity or enhancing the activity of non-selective cation channels. One study suggests that LVGCC were being opened in response to ROS-dependent voltage-gated K^+ channel (K_V) inhibition and subsequent depolarisation [58]. However this response may be specific to the pulmonary vasculature (section 7.2), while a number of other studies in systemic vessels suggests that K_V channel activity in VSM is enhanced by ROS, contributing to a pro-relaxant effect [350, 351, 366] (section 5.5). Alternatively, ROS are more likely to depolarise VSM through the opening of Ca^{2+}-activated Cl^- channels or TRP channels, which themselves also conduct Ca^{2+} [327]. In aortic VSMCs, GPCR agonists induce inward Ca^{2+} currents and contraction that are dependent on both TRPC6 and NOX (probably 1 or 2) and these effects are mimicked by exogenous H_2O_2 [131, 367]. In rat pulmonary artery, H_2O_2 enhances TRPC1/Stim1/Orai1-dependent Ca^{2+} influx [368], and Ca^{2+}-sensing receptor/TRPC6-dependent Ca^{2+} influx [369], while in renal afferent arterioles superoxide activates Ca^{2+}-activated Cl^- channels to enhance myogenic tone [327]. As with LVGCC, ROS are likely to be activating these channels indirectly via intracellular signalling, perhaps dependent on PLC/DAG and/or PKC [327, 367, 370]. There is also some evidence, albeit primarily from cardiac muscle, that the plasma membrane Ca^{2+} ATPase is inhibited by ROS, a pro-contractile effect [13], while the Na^+/Ca^{2+} exchanger may be activated, opposing this effect [13, 371].

4.3. NOX/ROS-dependent Ca^{2+} store handling. Most VSM GPCR are coupled to Gq to stimulate production of IP_3, so will trigger Ca^{2+} release from the sarcoplasmic reticulum via the IP_3 receptor (IP_3R). However, they may also open IP_3R indirectly via NOX-derived ROS, since both exogenous and NOX-derived superoxide in VSM and endothelium appear to directly sensitize IP_3R to IP_3, increasing

the rate of stimulated Ca^{2+} release [372, 373]. Alternatively, H_2O_2-induced contractile responses in aorta and pulmonary artery are partially dependent on sarcoplasmic reticulum Ca^{2+} release via ryanodine sensitive channels (RyR) [326, 344]. In addition, many GPCR agonists induce RyR-dependent Ca^{2+} release, usually secondary to nearby Ca^{2+} influx or release by other sarcoplasmic reticulum channels such as the Two-Pore channel or IP_3R (Ca^{2+}-induced Ca^{2+} release). Of the three subtypes (RyR1, 2 and 3), RyR1 is probably pro-relaxant in systemic blood vessels ([374], but may be pro-contractile in others [326], whereas RyR2 is universally considered pro-contractile [375]. All three subtypes are known to be directly ROS sensitive, containing multiple reactive cysteine residues that are important for channel activity [13, 376]. However, the functional significance of this has not been demonstrated in VSM. Instead Ang II-induced, RyR-mediated, sarcoplasmic reticulum Ca^{2+} release in renal afferent arterioles and pulmonary artery is indirectly dependent on NOX2 and also perhaps NOX4, via ROS-dependent production of CD38-derived cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP), activators of the RyR and Two-Pore channel, respectively [377-379]. In addition, vascular RyR2 may also be activated by ROS-induced dissociation of the inhibitory FKBP12.6 protein [375]. Ca^{2+} is pumped back into the sarcoplasmic reticulum by the sarcoplasmic reticulum Ca^{2+} ATPase (SERCA), and there is evidence that SERCA activity is suppressed by direct irreversible cysteine oxidation [13, 380], thus prolonging increases in $[Ca^{2+}]_i$. Recent evidence suggest that this oxidation of SERCA, albeit in cardiac muscle, may be mediated by NOX4-derived ROS in the sarcoplasmic reticulum [381]. It is not known whether NOX5 expressed in the sarcoplasmic reticulum contributes the regulation of Ca^{2+} homeostasis. The role of NOX-derived ROS in VSM Ca^{2+} influx and Ca^{2+} release signalling pathways is summarised in Figure 4.

4.4. NOX/ROS-dependent, Ca^{2+}-independent contractile signalling. In parallel with Ca^{2+}-dependent activation of MLCK, there is also concomitant inhibition of MLCP which further enhances MLC_{20} phosphorylation and contraction independently of a change in $[Ca^{2+}]_i$, a process that is often termed 'Ca^{2+}-sensitization' [382]. In line with this, exogenous ROS induce contraction in permeabilised, Ca^{2+}-clamped rat pulmonary artery [311, 326, 343, 351], and a high concentration of superoxide also does so in non-permeabilised arteries without raising $[Ca^{2+}]_i$ [311]. The main signalling pathway in VSM for inhibition of MLCP is phosphorylation of myosin phosphatase targeting subunit-1 (MYPT1) by Rho-activated kinase (ROCK) [382, 383]. ROCK is a serine threonine kinase that exists in a folded inactive form in the cytosol until activated by the monomeric G-protein RhoA [384]. Prior to this, RhoA is activated by the exchange of bound GDP for bound GTP, a process usually catalysed by Rho-specific guanine nucleotide exchange factors (RhoGEFs).

Contractions elicited by exogenous superoxide in rat aorta and pulmonary artery are blocked by inhibition of ROCK and are independent of dismutation to H_2O_2 [114, 311, 339, 351]. Sub-cellular translocation of ROCK, ROCK-mediated phosphorylation of MYPT1 and subsequent MLC_{20} phosphorylation, are all induced by exogenous superoxide [176, 311, 339, 351]. Although there is no evidence that Rho-kinase is directly ROS-sensitive, exogenous superoxide causes cytosolic to membrane translocation of RhoA in rat aorta and pulmonary artery [174, 176, 339]. This may be because RhoA is directly ROS sensitive, as it contains reactive cysteine residues that when oxidised

mimic the effect of RhoGEF binding, lowering the affinity for guanine nucleotides and triggering exchange of GDP for GTP [385, 386]. Alternatively, ROS may activate RhoA and ROCK upstream of RhoGEF activation, since superoxide induced contraction in rat pulmonary artery is suppressed by RhoGEF inhibition [174]. Such a pathway probably involves tyrosine phosphorylation of the RhoGEF by ROS-sensitive NRTKs. Src is a prime candidate, being directly ROS-sensitive and important for GPCR and superoxide-induced RhoGEF translocation, RhoA translocation and/or MYPT1 phosphorylation in rat afferent arterioles and pulmonary artery [106, 133, 174, 272, 311]. Src is also known to activate RhoGEFs indirectly via other tyrosine kinases FAK, PYK2 or JAK2 [145, 313, 314]. Once activated, RhoA also directly promotes actin polymerisation and, via ROCK and LIM kinase, inhibits the actin severing protein cofilin [387]. There are two main physiological contractile stimuli that may activate RhoGEFs and subsequently activate RhoA and ROCK via NOX-derived ROS. GPCR directly activate RhoGEFs via $G_{12/13}$, but also activate NOX1 or NOX2, and the superoxide produced will activate Src, PYK2 and JAK2 to promote RhoGEF activation [174, 295], as described above. Stretch induces integrin engagement with the extracellular matrix, FAK auto-phosphorylation and subsequent RhoGEF phosphorylation [270]. This 'outside-in' FAK activation is also associated with activation of NOX4/Poldip2 [229]. One study shows that NOX4/Poldip2-depedent H_2O_2 production induced by integrin engagement results in direct cysteine oxidation of filamentous actin and this promotes subsequent maturation of focal attachments [49]. Another study has shown that NOX4 derived ROS oxidise integrin $\alpha7\beta1$ and that this induces extracellular matrix binding [388] and potentially additional activation of FAK, suggesting a two-way relationship between integrin engagement and NOX4 activation which may strengthen the transmission of contractile force [389]. This response to stretch is of particular importance to the role of NOX in myogenic contraction (section 4.6). In line with the importance of RhoA/ROCK signalling in GPCR-induced VSM contraction and the role of NOX in activating ROCK, GPCR-induced translocation/activation of RhoGEFs/RhoA and/or MYPT1/MLC_{20} phosphorylation in rat afferent arterioles, and mesenteric, tail and pulmonary arteries, are inhibited by non-specific antioxidants, SOD-mimetics or NOX blockers [106, 114, 133, 135, 174].

Unlike superoxide, some reports suggest that exogenous H_2O_2 induces contraction in a number of vascular beds that is insensitive to ROCK inhibition and is not associated with RhoA or ROCK translocation or phosphorylation of MYPT1, and is even independent of MLC_{20} phosphorylation [311, 326, 343, 346, 347]. Indeed H_2O_2 actually suppresses basal MLC_{20} phosphorylation at the main MLCK target site (Ser19) [326, 343]. Instead, in permeabilised, Ca^{2+}clamped tissues H_2O_2 causes a contraction that is sensitive to inhibition of PKC [326, 343]. In parallel to ROCK-dependent MLCP inhibition via MYPT1, PKC, which is itself directly ROS sensitive [176, 321, 322], also inhibits MLCP through phosphorylation of the inhibitory protein, C-kinase potentiated protein phosphatase-1 Inhibitor-17 (CPI-17) [135, 382, 390]. In addition, there are two further routes through which ROS, with the involvement of PKC, could influence contractile force, and both involve actin polymerisation. Firstly, there is heat-shock protein-27 (HSP27) a chaperone which prevents actin polymerisation, unless phosphorylated and inhibited by p38 MAP-kinase via MAP-Kinase activated protein kinase [315]. Secondly, there is caldesmon, which inhibits actin-myosin interaction unless it is phosphorylated and inhibited by ERK MAP-kinase [391]. ERK and p38 MAP-kinases are both indirectly activated by ROS via

upstream PKC-mediated MEK phosphorylation [156, 194, 392]. MEK is also activated via oxidative inhibition of upstream inhibitory PTPs [268], oxidative activation of Ras [393] or Src-dependent RTK transactivation and recruitment of GRB2, Ras and Raf. In line with these signalling pathways being pro-contractile in VSM, constrictions induced by exogenous H_2O_2 or NOX-dependent GPCR agonists or stretch are inhibited by blockers of either p38 or ERK MAP-kinases [103, 194, 283, 341, 352-354, 394, 395]. ROS-dependent, Ca^{2+}-independent contractile signalling pathways are summarised in Figure 5.

4.5. Role of NOX in GPCR-induced contraction. Having established the pro-contractile effects of ROS in VSM and the signalling pathways involved, this review now addresses the relevance of NOX-derived ROS to physiological vasoconstrictor stimuli, starting with GPCR agonists. These include agents involved in the control of mean arterial blood pressure and central control of regional blood flow (Ang II, noradrenaline) or local control of blood flow (ET-1, adenosine, 5-HT, prostaglandins, sphingolipids etc), all of which are known to activate NOX and stimulate ROS production (Table 1). Complementary to this, a variety of studies in isolated arteries from various vascular beds have utilised non-selective antioxidants, pharmacological manipulation of NOX and antioxidant enzymes, to examine the role of NOX in VSM contraction (Table 2). For example, Ang II in isolated aorta, mesenteric, cerebral and pulmonary arteries and renal afferent arterioles [89, 99, 104, 105, 133, 137, 325, 335, 377, 378], catecholamines in aorta, tail artery and renal arteries [131, 135, 137, 226], ET-1 in mesenteric and pulmonary arteries [66, 114], eicosanoids in mesenteric, coronary and pulmonary arteries [66, 140, 146, 148, 174], adenosine in aorta [116] and 5-HT in pulmonary artery [138], all cause constriction in part via NOX and ROS. A couple of these studies have also examined the role of NOX-derived ROS in renal haemodynamics *in vivo*. Infusion of an antioxidant or NOX inhibitor enhances baseline renal blood flow [137]. Such infusion also reverses the inhibition of renal blood flow by Ang II or noradrenaline and antagonises the concomitant increase in renal vascular resistance [133, 137]. Through the use of knockout mice or specific inhibitors, these responses have been attributed to NOX1 or NOX2 ($p47^{phox-/-}$)[104], NOX1 alone ($NOX1^{-/-}$) [89, 138, 148], NOX2 alone (gp91ds-tat, $NOX2^{-/-}$) [133, 140], or Poldip2/NOX4 ($Poldip2^{+/-}$) [226]. The role of NOX in Ang II induced vasoconstriction is of particular importance to the study of NOX involvement in hypertension (section 6).

4.6. Role of NOX in myogenic, pressure/stretch-induced contraction. In a number of vascular beds, arterioles constrict in response to increased perfusion pressure (myogenic tone) in order to maintain adequate organ blood flow despite changes in MAP and prevent tissue damage through excessive flow. This is important in the cerebral [325, 396] and renal circulations [397], but has been studied most extensively in the renal afferent arteriole, where NOX-derived ROS play an important role. In the perfused pressurised afferent arteriole, endogenous superoxide production is increased by raising perfusion pressure [104, 230, 327, 334] (Table 1) and the response is suppressed in $p47^{phox-/-}$ mice, suggesting the involvement of NOX1 or NOX2 [104, 230] or in $Poldip2^{+/-}$ mice suggesting the additional involvement of NOX4 [230] (Table 2). There is limited evidence that stretch activates NOX in other vascular beds. Myogenic tone is of marginal importance in coronary arteries, and pulmonary arteries do not generate force in response to stretch alone, but passive stretch does influence force generated in response to other stimuli. For example, depolarisation induced contraction in bovine pulmonary

and coronary arteries is enhanced by previous application of supra-physiological stretch and this enhancement appears to be dependent on NOX-derived ROS [194, 353].

Exactly how NOX1 or NOX2 are activated by stretch in VSM is unknown, but it is reasonable to speculate that a key step is the integrin-mediated activation of Src and FAK following stretch-induced binding of integrins with the extracellular matrix, the so-called 'outside-in' activation pathway, perhaps via interaction with transmembrane PTPs [398]. This may also involve subsequent activation of PKC. Indeed, one study in femoral artery suggests that stretch-induced ROS production requires PKC-dependent phosphorylation of $p47^{phox}$ [162]. Like GPCR-induced responses, myogenic contraction involves both Ca^{2+} influx (primarily via LVGCC) and RhoA/Rho-kinase mediated Ca^{2+} sensitization [325, 327, 355, 399] and NOX-derived ROS will contribute to these, perhaps via stretch-induced Src- and PKC-dependent LVGCC phosphorylation [363-365] or RhoGEF phosphorylation [145, 313, 314]. An important role for NOX1 or NOX2-derived superoxide in myogenic contraction is suggested by the inhibitory effects of antioxidants, pharmacological NOX inhibitors and $p47^{phox}$ knockout in isolated perfused renal afferent arterioles [99, 104, 230, 325, 334]. A pathway for NOX4 activation by integrin engagement which may contribute to myogenic tone can be inferred from the known association between Poldip2/NOX4, integrins, FAK and the actin cytoskeleton, and its role in integrin-induced VSM focal adhesion turnover [47, 49, 107, 228, 229] (Figure 5, and see also section 6.3 on the role of NOX in VSM migration). However, contrary to this and contrary to the proposed role for NOX4 in Ca^{2+}-independent contraction via actin polymerisation, NOX4-derived H_2O_2 appears to be antagonistic of NOX1- or NOX2-derived superoxide and inhibitory of myogenic tone, rather than contributing to it. Note that afferent arteriole myogenic tone is also inhibited by over-expressing SOD and suppressed with PEG-SOD or enhanced in $SOD^{-/-}$ mice, strongly suggesting that superoxide, not H_2O_2, is the specific ROS mediating this response [99, 230, 334]. Conversely, inhibiting NOX4-derived H_2O_2 with PEG-catalase or in $Poldip2^{+/-}$ mice has the opposite effect, enhancing myogenic tone, indicating a pro-relaxant effect of H_2O_2 [230, 327]. Consistent with this, exogenous superoxide enhances myogenic tone in cerebral arteries and renal afferent arterioles [325, 327] while exogenous H_2O_2 has a mixed dose-dependent effect on myogenic tone in renal afferent arterioles, enhancing the response at low concentrations but causing relaxation at higher concentrations [334]. The pro-relaxant properties of ROS, and H_2O_2 in particular, are discussed further in the next section.

5. Role of NOX in VSM relaxation.

Exogenous superoxide, as generated extracellularly by xanthine/xanthine oxidase or intracellularly by LY83583 or menadione, relaxes rat aorta and mesenteric and femoral arteries pre-stimulated with vasoconstrictor GPCR agonists [311, 333, 351] and suppresses rat cerebral myogenic auto-regulation *in vivo* [396]. Superoxide may do this independently of its dismutation to H_2O_2 [311], but exogenous H_2O_2 also clearly causes relaxation in a range of systemic vascular preparations pre-stimulated with a range of vasoconstrictor GPCR agonists [336, 350, 354, 400-407] or pressure-induced myogenic tone [327, 334, 354, 408-411] and increases canine coronary blood flow *in vivo* [336] (Table 3). Thus, perhaps paradoxically, as well as mediating vasoconstriction, endogenous ROS also mediate vasodilation, often in the same vascular beds. This section discusses the evidence for a vasodilatory

action of NOX-derived ROS in response to normal physiological vasodilatory stimuli, focussing on the regulation of its production, its role in modulating endothelial function and its target pro-relaxant effector pathways in the smooth muscle.

5.1. Activation of Endothelial NOX. Physiological stimuli that trigger VSM relaxation and increase blood flow include vasodilatory GPCR agonists such as acetylcholine (from parasympathetic efferent nerves), histamine and bradykinin (local inflammatory mediators) and others, as well as flow/shear-stress. A response to shear-stress is important in some vascular beds for accommodating increased flow in circumstances where cardiac output and MAP are increased, such as muscle during exercise. Conversely, in tissues where relatively constant flow is desirable such as brain and kidney, flow-induced dilation balances and is balanced by pressure-induced myogenic contraction [230, 334, 412]. GPCR-induced and flow-induced relaxation are both endothelium-dependent and not wholly dependent on eNOS, therefore the ROS contributing to this relaxation are likely to be endothelial in origin and may act in parallel with or even influence eNOS and EDHF. As in VSM, NOX isoforms 1, 2, 4 and 5 are all expressed in the vascular endothelium [53, 54, 215, 234, 413-415] and are likely sources of the ROS contributing to or influencing these relaxation responses. Indeed, acetylcholine, histamine, bradykinin and flow all induce NOX-derived ROS production in vascular tissues and cultured endothelial cells [237, 402, 405, 408, 414, 416-418, 418-421] (Table 1). Vascular relaxation responses in isolated arteries and cerebral blood flow are improved by non-selective NOX inhibitors [421, 422], and several studies have examined the role of specific endothelial NOX isoforms in endothelium-dependent relaxation (Table 2). So far, the only unambiguous evidence for a pro-relaxant isoform is for NOX4. Femoral artery relaxation responses are suppressed in NOX4 knockout [52] while coronary perfusion pressure is depressed by over-expression of NOX4 [215]. Conversely, deletion of NOX1 appears to improve endothelium-dependent relaxation in isolated aorta or pressurised mesenteric arteries [423, 424] while expression of NOX5 in mouse vasculature appears to suppress it [66]. For NOX2, there is conflicting evidence. Some studies using selective inhibition of NOX2 or NOX2 knockout suggest that NOX2-derived superoxide antagonises endothelium-dependent relaxation [418, 425, 425], while other suggest that it supports endothelium-dependent relaxation in response to both flow and acetylcholine [418]. As discussed below, these differences may relate to the different pathways through which the four NOX isoforms respond to stimuli acting on the endothelium and the nature of their effects within the endothelium, but perhaps most significantly, the fact that NOX4 produces H_2O_2, not superoxide.

Activation of NOX1, NOX2 and NOX5 isoforms by GPCR agonists in endothelium is essentially the same as in VSM and has already been discussed, while the exact mechanism of NOX4 activation by GPCR in endothelium remains unclear (section 2). NOX activation by laminar flow/ shear-stress on the other hand is specific to the endothelium because only the endothelium is in direct contact with flowing blood. Endothelial cells possess a mechanical flow sensor or 'mechanosome', a protein complex composed of platelet-endothelial cell adhesion molecule-1 (PECAM-1) and the adapter protein VE-cadherin, associated with caveolin in caveolae. In response to changes in flow, this complex transactivates the type-2 VEGFR which then acts as a platform for the activation of PI3K [426], PKC-δ

[427], Src [428] and Rac1 [429], all of which contribute to the assembly and activation of NOX1 and NOX2 (see Figure 1 for more specific details). Secondly, VEGFR activation also triggers closure of K_{ATP} channels, similarly associated with caveolae [430, 431]. It is believed that the resultant endothelial depolarisation opens T-type Ca^{2+} channels, thus raising endothelial $[Ca^{2+}]_i$ [432, 433]. This initial increase in $[Ca^{2+}]_i$, as well as having downstream actions, may activate PKC-α or -β, further contributing to NOX1/2 activation. It may also directly activate NOX5, although this has not been demonstrated experimentally. In the endothelium, caveolin-1 appears to constitutively suppress NOX1 and NOX2 activity in the short term and suppress their expression in the long term [48, 234, 430, 431] and it is likely that HSP90 relieves this suppression by displacing caveolin-1 from the oxidase catalytic subunit [234], in a manner similar to that previously described for the activation of eNOS [434]. However, it should be noted that several earlier publications on the relationship between NOX and caveolin (not cited here) have since been retracted, so later studies should perhaps be interpreted with caution. NOX4 is also activated by sustained laminar shear-stress in endothelium, possibly explaining the high baseline levels of NOX4-derived endothelial H_2O_2 *in vivo* [291]. However, it is unclear how laminar shear-stress activates NOX4 since it is not located in caveolae [23] and the activation mechanism must differ from that described for VSM because integrins are not expressed on the apical surface of endothelium. Once activated, endothelial NOX generates H_2O_2 (NOX4) or superoxide (NOX1, 2 and 5) within the cell, where it contributes to the regulation of vascular tone through three main pathways: interaction with eNOS to regulate NO· production, both positively (H_2O_2) and negatively (superoxide), opening of endothelial K^+ channels as part of an endothelium-derived hyperpolarisation response, and diffusion (H_2O_2 only) to the VSM to cause relaxation directly, primarily via opening of VSM K^+ channels.

5.2. Cross-talk between NOX and eNOS in the endothelium. A key mediator of VSM relaxation is endothelium-derived NO·, produced by eNOS through the transfer of an electron from NADPH to L-arginine in the presence of tetrahydrobiopterin (BH_4) [435]. The initial trigger for eNOS dimerization and activation is usually an increase in $[Ca^{2+}]_i$, either via sarcoplasmic reticulum Ca^{2+} release in response to GPCR agonist binding (eg acetylcholine, bradykinin and adenosine), or Ca^{2+} influx triggered by laminar shear-stress, as described above. NO· diffuses to the smooth muscle to cause relaxation, primarily via the activation of guanylate cyclase, production of cGMP and activation of protein kinase G (PKG), resulting in opening of K^+ channels. Classically, endothelial or smooth muscle derived superoxide is considered a major opponent of NO· mediated relaxation because it will chemically react with NO·, generating peroxynitrite ($ONOO^-$) which is a relatively weak vasodilator [362, 436]. This is supported by the fact that applying or over-expressing SOD supports NO· mediated relaxation, while knocking out SOD has the opposite effect [99, 437]. Also, superoxide has the potential to trigger uncoupling of eNOS by oxidising BH_4, thus forcing eNOS to make more superoxide instead of NO· [413, 435]. On the other hand, endogenous SOD normally dismutates much of the endothelial superoxide into H_2O_2, which is pro-relaxant [402, 404, 410, 438], and NO· may indirectly support H_2O_2 production over that of superoxide through selective inhibitory S-nitrosylation of $p47^{phox}$ (NOX1, 2) and of NOX5 [439, 440], but leaving NOX4-derived H_2O_2 unaffected [52, 440]. There is also evidence that endothelial ROS more directly promote eNOS activity and NO· production, with either NOX2-derived

superoxide [414, 418, 420] or NOX4-derived H_2O_2 [209, 291, 441], activating several protein kinases, including CAMK-II, p38 MAPK and FAK, either sequentially or in parallel, resulting in eNOS phosphorylation and further enhanced NO· production [209, 291, 442]. Interestingly, it is also apparent that sustained periods of physiological laminar pulsatile shear-stress also suppress $gp91^{phox}$/NOX2 expression, thus reducing basal superoxide production [414, 443]. It also upregulates eNOS expression [444], while at the same time it promotes NO· dependent down-regulation of NOX2 expression [420].

5.3. Role of NOX-derived ROS in endothelial hyperpolarisation. The second pathway through which ROS can promote endothelium-dependent relaxation from within the endothelium is by opening the intermediate and small conductance Ca^{2+}-activated K^+ channels (IK_{Ca}/SK_{Ca}) on the endothelium to trigger hyperpolarisation [405, 408], which then spreads to the VSM via myo-endothelial gap junctions [406, 445] or by the effluxed K^+ diffusing to the VSM and activating VSM inward rectifier K^+ channel (K_{IR}) and Na^+/K^+ATPase, which then hyperpolarises the VSM [446, 447]. There is little evidence that IK_{Ca} or SK_{Ca} are directly ROS-sensitive, but they are activated by an increase in endothelial $[Ca^{2+}]_i$ which can be induced by H_2O_2. Endothelial H_2O_2 may elevate $[Ca^{2+}]_i$ by promoting Ca^{2+} release from the sarcoplasmic reticulum or by triggering endothelial Ca^{2+} influx, and there is evidence for both. As in VSM, endothelial Ca^{2+} release occurs through a combination of IP_3R and RyR efflux pathways [448, 449] and, also as in VSM, these may be enhanced by H_2O_2 [225, 450]. This idea is supported by a study in isolated rabbit iliac artery, where exogenous H_2O_2 enhances the release of endothelial sarcoplasmic reticulum Ca^{2+} and relaxation triggered by pharmacological SERCA inhibition, and the enhanced relaxation is suppressed by blockers of IK_{Ca} / SK_{Ca} and myo-endothelial gap junctions [406]. Alternatively, the endothelial transient receptor potential ankyrin-1 (TRPA1) is also directly ROS-sensitive [451, 452]. TRPA1 has a large conductance and may itself facilitate sufficient endothelial Ca^{2+} influx to activate co-localised SK_{Ca} and IK_{Ca} on the endothelial basolateral surface [453-456]. One additional study provides evidence supporting a role for NOX-derived H_2O_2 in endothelial hyperpolarisation. Here, enhanced ACh-induced relaxation induced by endothelium-selective over-expression of NOX4 in isolated aorta was normalised by catalase, KCl and blockers of SK_{Ca} and IK_{Ca}, suggesting that the extra H_2O_2 generated was acting within the endothelium to promote endothelial hyper-polarisation [215]. Therefore, H_2O_2 may play an accessory role in endothelium-dependent relaxation as a promotor of endothelial-VSM electrical coupling.

5.4. NOX-derived H_2O_2 as a diffusible hyperpolarising factor. EDHF was originally defined to describe an unknown factor or factors that may be responsible for the component of endothelium-dependent hyperpolarising vasodilation not due to NO· or prostacyclin [457, 458]. We now know that EDHF probably comprises no single factor but a range of diffusible factors or processes coupling endothelial hyperpolarisation to smooth muscle hyperpolarisation and relaxation [446, 459]. In addition to contributing to endothelial hyperpolarisation and electrical coupling between endothelium as described above, H_2O_2 may itself be one of these proposed factors that diffuses to the VSM and causes hyperpolarisation [410]. Flow/shear-stress and vasodilatory GPCR agonists such as acetylcholine, bradykinin, histamine, substance P and adenosine induce hyperpolarisation and dilation of a wide

range of isolated resistance-sized systemic arteries/arterioles from various mammalian species that is suppressed when SOD is inhibited or knocked out or when catalase is applied, and is restored by applying SOD or SOD mimetics [52, 102, 117, 139, 215, 400-402, 404-406, 408, 410, 411, 418, 421, 425, 437, 438, 460-463]. Thus, it is presumably H_2O_2 not superoxide, that plays the major role in relaxation responses to physiological vasodilator stimuli, even though exogenous superoxide is also capable of relaxing VSM. This may be partly because superoxide scavenges NO· but H_2O_2 does not, and partly because H_2O_2 is more freely diffusible and membrane permeable than superoxide. In many of the studies implicating H_2O_2 as an EDHF, the principle source of endothelial H_2O_2 is not clearly defined, although a role for NOX can often be inferred. Two studies have directly linked NOX to EDHF, suggesting that either inhibition of NOX2 or knockout of NOX4 will remove the PEG-catalase inhibitable component of adenosine-induced relaxation of isolated rat cerebral arterioles [117] or of flow-induced femoral dilation *in vivo* [52]. One alternative source is uncoupled eNOS [402, 461].

5.5. Pro-relaxant targets for ROS in VSM. In arteries pre-stimulated with constrictor GPCR agonist or myogenic tone, the EDHF component of relaxation induced by vasodilatory GPCR agonists or flow after blockade of eNOS or prostacyclin production, is inhibited by a depolarising KCl solution to remove the influence of K^+ channels on VSM membrane potential [402, 405, 411], suggesting that EDHF ultimately relaxes the VSM by opening K^+ channels, triggering hyperpolarisation, closing LVGCC and lowering $[Ca^{2+}]_i$. In similarly pre-constricted vessels, the relaxation induced by either H_2O_2 or superoxide is also inhibited, or even turned into a contractile response by KCl, supporting the hypothesis that H_2O_2 is an EDHF [148, 336, 341, 342, 350-352, 354, 402, 405, 407, 408, 411].

VSM express a range of K^+ channels including, most notably, a number of different K_V channels and the large conductance Ca^{2+} activated K^+ channel (BK_{Ca}). However, there appears to be conflicting evidence over which of these two types of channel are the most important in mediating VSM hyperpolarisation responses to physiological vasodilatory stimuli. Certainly there is conflicting evidence over which is important in mediating the hyperpolarising and pro-relaxant effects of exogenous H_2O_2 or superoxide. Some studies show that superoxide or H_2O_2-induced relaxations of GPCR-mediated or myogenic contractions in coronary and mesenteric arteries or renal afferent arterioles are prevented by selective pharmacological inhibition of K_V channels (eg. 4-aminopyridine, 4-AP, linopirdine), but not of BK_{Ca} channels (eg. TEA, iberiotoxin) [327, 336, 350, 351, 366, 407, 464], or that H_2O_2 enhances coronary blood flow in dogs *in vivo* and this is blocked by 4-AP [336]. In line with this, at the single cell or single channel level, a few studies show that exogenous superoxide (10μM LY83583) and somewhat high concentrations of H_2O_2 (1-10mM) both activate 4-AP sensitive K_V currents in isolated coronary and mesenteric VSMCs [350, 351, 366, 407]. K_V channels expressed in VSM may be directly ROS sensitive since the current enhancement by H_2O_2 in coronary arterial VSMCs is prevented by thiol alkylation [366] and $K_V2.1$ is modified by direct S-glutathionylation (oxidised glutathione) in mesenteric artery VSMCs to increase its voltage sensitivity [407], or it may be indirect via MAP-kinase dependent phosphorylation [409].

Curiously, despite all the evidence described above that K_V channels are preferentially activated by ROS and contribute to ROS mediated relaxation, when examining hyperpolarisation and VSM relaxation induced by physiological vasodilatory stimuli acting via endogenous H_2O_2, the majority of evidence points towards BK_{Ca} as the likeliest target channel because acetylcholine and flow both induced relaxation in coronary or mesenteric arteries that is suppressed by inhibitors of BK_{Ca} channels (TEA, TBA, iberiotoxin, paxilline) [402, 403, 410, 411, 460, 462, 462]. This is also supported by additional studies showing that exogenous superoxide (X/XO) and physiological concentrations of H_2O_2 (1-100μM), as well as perfusate from mesenteric arteries containing endogeous H_2O_2, activate single channel BK_{Ca} currents in canine cerebral artery or human coronary artery VSMCs [396, 403, 410, 460]. Unlike for K_V channels, there is limited evidence for direct oxidative activation of BK_{Ca}. Although the human BK_{Ca} channel reportedly possesses a disulphide switch in non-vascular cells [465], there is no evidence to support its direct oxidative modification in vascular cells, indeed physiological concentrations of exogenous H_2O_2 (<100 μM) only effectively activates BK_{Ca} in cell-attached patch-clamp configuration, indicating the requirement of intracellular signalling [403, 410]. It is more likely that it requires oxidative activation of kinases that phosphorylate and open these channels, particularly PKGIα, either directly (independently of cGMP) [327, 400, 401, 403, 466], or via oxidative activation of guanylate cyclase which generates cGMP, the main activator of PKG [467, 468]. In addition to opening K^+ channels, there is some evidence that under certain circumstances, ROS and/or NO· may promote relaxation by inhibiting L-type Ca^{2+} channels through direct thiol sulfonylation or S-nitrosylation [359-362]. However, it is unclear how this mechanism can operate and be regulated alongside the better characterised indirect opening of L-type Ca^{2+} channels by ROS-dependent phosphorylation or membrane depolarisation (section 4.2). Finally, $[Ca^{2+}]_i$ may be lowered to promote relaxation through glutathionylation and activation of VSM SERCA [380]. All of the interactions between endothelial superoxide, H_2O_2 and NO·, their contribution to EDHF and VSM relaxation and the effector pathways are summarised in Figure 6.

A big question remains though: how ROS can have opposing effects, contraction and relaxation, in the same tissue or even the same cell within that tissue, and how the final net response is obtained. This presumably relates to the relative sensitivity of the various ROS effector pathways to ROS and therefore the concentration of ROS applied, but also to the relative expression levels of components of those pro-contractile and pro-relaxant pathways and their possible co-localisation with NOX in the endothelium and VSM. Differential effects of superoxide vs. H_2O_2 within the cell may also be important, and therefore the relative activities and distributions of SOD and catalase. Clearly, the nature of the pre-constriction influences the outcome, not only by altering the balance between LVGCC and K^+ channel activity but also the contributions of VSM sarcoplasmic reticulum Ca^{2+} release and Ca^{2+} sensitization pathways, both of which are also ROS sensitive but minimally influenced by membrane potential or NO·.

6. Role of NOX-derived ROS in hypertension.

Hypertension is a persistent increase in baseline MAP, which if uncorrected will result in cardiac remodelling and ultimate heart failure. There are various causes, including over-activation of the

renin-angiotensin-aldosterone system and sympathetic nervous system, various genetic factors and environmental/life-style factors, such as a high salt diet, excess body weight and smoking [469-472]. Regardless of cause, disease progression involves several underlying mechanisms, including inflammation, smooth muscle hyper-contractility coupled with impaired endothelial function, smooth muscle proliferation and migration. A common feature of all these processes and the resultant hypertension is oxidative stress, as indicated by elevated levels of malondealdehyde and F2 isoprostanes, resulting from excessive ROS production, reduced NO· availability and impaired antioxidant defences [113, 415, 470, 472-478]. A clear causative link between oxidative stress and hypertension remains a matter of debate, especially in humans where there also remains uncertainty over the therapeutic benefit of antioxidants [469, 470, 472, 474, 476, 477, 479-482]. However, such a link is well characterised in multiple animal models of hypertension, such as the spontaneously hypertensive rat (SHR), renovascular hypertension, deoxycorticosterone acetate (DOCA) salt rat, obese rat and the Ang II-infused rat or mouse. These have been used to examine the mechanisms underlying oxidative stress and its role in the pathophysiology of hypertension. Throughout earlier sections of this review are detailed the various cellular target signalling pathways for NOX-derived ROS that contribute to the normal functioning of the vasculature, both pro-contractile and pro-relaxant. In this section will be outlined evidence for how these pathways are altered in oxidative stress to promote a hyper-contractile state, how excess ROS induce vascular remodelling through stimulation of VSM growth, proliferation and migration and fibrosis, and briefly, the role of NOX/ROS in vascular inflammation.

6.1. Expression of NOX isoforms/subunits in hypertension. Individually, NOX isoforms may contribute to VSM hyper-contractility or remodelling in response to hypertensive stimuli through altered activity, expression or both [80]. NOX1 and 2 are both upregulated in SHR compared to Wistar-Kyoto control (WKY) rats [415, 483-485]. The regulation of subunit NOX expression by long-term treatment of vascular cells/tissues with mitogenic GPCR agonists and growth factors/cytokines has been reviewed extensively elsewhere [23, 474, 486]. Examples from rat or mouse models include expression of NOX1, NOX2, $p22^{phox}$, $p47^{phox}$ and $p67^{phox}$ in VSM being enhanced by Ang II infusion [26, 40, 45, 82, 423, 423, 473, 483, 487-491], NOX1 being enhanced by PDGF [40], NOX1 by stretch [492] and NOXA1 by TNF-α [126]. There is some disagreement over whether NOX4 is also upregulated in SHR, with several studies saying it is [70, 483, 485, 493, 494] and some saying it is not [415]. Regulation of NOX4 expression by mitogenic and inflammatory stimuli in hypertension is also contentious. Several reports suggest NOX4 is upregulated by GPCR agonists and inflammatory mediators in VSM [47, 120, 263, 423, 489, 494, 495] while a few others suggest it is down-regulated by Ang II and PDGF [40, 45, 473]. However, a recent study suggests that, either in SHR or during Ang II infusion, expression of NOX4 in VSM is enhanced, and this occurs by the suppression of B-cell lymphoma 6 (BCL6) [494]. BCL6 itself normally suppresses NOX4 expression, reduces oxidative stress and impairs aortic wall thickening [494]. NOX5 expression is enhanced by Ang II, ET-1 and inflammatory mediators such as TNF-α in human VSMCs *in vitro* [57, 496]. It is not clear whether native NOX5 expression is altered in VSM in hypertensive humans but it is upregulated in renal proximal tubule cells [497]. There is limited evidence that NOX polymorphisms are associated with hypertension risk in humans [470]. $p22^{phox}$ polymorphisms show

either a protective association or an increased risk [498] and seven identified polymorphisms in NOX5 result in reduced or absent enzyme activity [499]. See Figure 7 for a summary of all the factors influencing NOX expression in hypertension.

6.2. Impaired NO·-dependent relaxation and enhanced contractility. In SHR, amplitude of phenylephrine-induced contraction in isolated aorta is enhanced while acetylcholine induced relaxation is impaired compared to WKY controls and these differences are normalised by antioxidants and NOX inhibitors (tiron, tempol, apocynin, VAS2870) or catalase [415, 423, 425, 489]. If there is an imbalance between ROS production and antioxidant defences, then excess superoxide in the vasculature will have two immediate effects. Firstly, it will react with NO· in an uncontrolled way, resulting in the production of excess $ONOO^-$ and secondly it will trigger uncoupling of eNOS through oxidation of BH_4, forcing eNOS to make superoxide instead of NO·. Excessive ROS, particularly hydroxyl radical, may also directly inactivate guanylate cyclase [484, 500]. Hypertensive agents such as Ang II and TXA_2 promote eNOS uncoupling [5, 6, 66, 414, 422, 435, 501]. The degree to which this eNOS uncoupling impairs agonist-induced relaxation will also depend on how much of the superoxide produced is dismutated to H_2O_2 in the endothelium, and this will depend on the expression and location of SOD, which may or may not be down-regulated [99, 422]. The net effect will be impaired NO· mediated relaxation via K^+ channel closure and VSM depolarisation [425]. However, these pathways do not solely account for the hyper-contractile phenotype. Hypertension is also associated with a dysregulation of intracellular Ca^{2+} in the VSM [70, 502-504] and this is influenced by VSM oxidant stress [13, 70, 376]. There is evidence for both enhanced Ca^{2+} influx through LVGCC [503-507] and altered sarcoplasmic reticulum Ca^{2+} handling [506, 508, 509], but it perhaps remains to be clarified which of all the various ROS-dependent Ca^{2+} signalling pathways described in this review are predominantly upregulated in hypertension. For example, one study shows in mesenteric arteries from SHR, that H_2O_2 induces a greater LVGCC-mediated Ca^{2+} influx than in WKY control rats [348], while others suggests that one consequence of oxidative stress on $[Ca^{2+}]_i$ is that excessive cytosolic superoxide results in irreversible inhibition of smooth muscle SERCA, preventing the normal pro-relaxant, NO·-mediated S-glutathionylation of the ATPase [380] (section 5.5).

Hypertension is also associated with an increased activity of the RhoA/Rho-kinase signalling pathway and this also mediates both enhanced VSM contractile responses and VSM proliferation/migration [510-514]. As discussed earlier in this review, RhoA is both directly [385, 386] and indirectly ROS sensitive, so this enhanced activity could be NOX-dependent, perhaps via an upstream ROS-sensitive tyrosine kinase. For example, an Ang II-induced increase in MAP and aortic remodelling is suppressed in smooth muscle JAK2 knockout mice and part of the effect is ROS-dependent [192]. In carotid artery from SHR, enhanced agonist-induced contraction is inhibited by antioxidants and NOX inhibition while exogenous H_2O_2 causes a greater degree of Rho-kinase-dependent contraction than in WKY rats [515]. Antioxidants such as the putative SOD mimetic tempol and vitamins C and E lower blood pressure in various rodent models of hypertension [133, 516-520]. This effect is also accompanied by corrected endothelial dysfunction, particularly improved NO· activity and reduced agonist evoked ROS production, and normalised blood vessel contractility and vascular resistance [519]. SOD itself has a

similar restorative effect on endothelium-dependent relaxation [102, 425] and blood pressure [488], while knocking out SOD1 enhances the transient phase of Ang II-induced hypertension in mice, suggesting a specific role for superoxide in mediating the initial pressor response [99]. Taken as a whole, these data suggest that a key feature of hypertension is indeed a shift in the balance between pro-contractile and pro-relaxant effects of ROS contributing towards a hyper-contractile phenotype (Figure 7).

6.3. Vascular remodelling in hypertension. Vascular remodelling, resulting in thickening of the smooth muscle, narrowing of the lumen and increased vessel stiffness, is a key feature of hypertension, either as a primary cause of elevated MAP or as a secondary response to prolonged periods of elevated perfusion pressure. Either way, ROS are essential components of the signalling pathways involved in this process (Figures 7 & 8). For VSM proliferation, various stimuli and proximal ROS-sensitive kinases, including Src, CAMK-II and PKC, converge on ERK MAP-kinase via growth-factor transactivation and Ras [521], or via phosphorylation of Raf and/or MEK [522, 523] or via PYK2/JAK2 [524]. Once phosphorylated, ERK translocates to the nucleus to induce gene expression and cell division and ROCK may supporting this translocation [525]. In addition, there is the JAK/STAT pathway, a common route for inflammatory responses and gene expression. JAK2 is principally induced by cytokine receptor activation, but it is also activated by GPCRs to phosphorylate signal transducer and activator of transcription (STAT), triggering its translocation to the nucleus to regulate gene expression. JAK2 also stimulates ERK phosphorylation [526], possibly downstream of ROS-activated Src family kinases [188, 527], PKC-δ [170] or PYK2 [524]. PYK2 is activated by Ca^{2+}, often in response to induced sarcoplasmic reticulum Ca^{2+} release, triggered by IP_3 generated by PLC-β (GPCR coupled) or PLC-γ (RTK-coupled). p38 MAP-kinase is also activated by MEK in response to various mitogenic GPCR or RTK stimuli via NOX-derived ROS or by TNF-α via apoptosis signal-regulating kinase-1 (ASK-1) in VSM endosomes. The latter requires NOX1-dependent oxidative activation of ASK-1 [248, 252]. Finally, PI3K is a key stimulator of cell growth and cell survival, acting via AKT, mammalian target of rapamycin (mTOR), S6-kinase and eukaryotic translation initiation factor 4E-binding protein (4EBP) [528].

Cell migration requires a dynamic cell polarisation involving co-ordinated cycles of actin de-polymerisation and polymerisation at the trailing and leading edges. This is dependent on activation of small G-proteins RhoA, Rac and cdc42, as well as FAK, interaction with ILK and cross-talk with extracellular matrix [107, 529]. ROS-activated CAMK-II may facilitate VSMC migration by activating the matrix metalloprotease MMP9 [328, 530]. At the leading edge of the migrating cell, actin depolymerisation is required for the formation of new actin fibres and this is achieved through the ROS-dependent de-phosphorylation and activation of cofilin by the PTP slingshot-1L following NOX4-dependent oxidation of 14-3-3 [531, 532]. Rho and Rac are often activated by migratory stimuli via tyrosine phosphorylation of their respective guanine nucleotide exchange factors, by Src, FAK, PYK2 or JAK2 and once activated they collectively promote actin reorganisation [145, 189, 190, 192, 313, 314]. Poldip2 may facilitate activation of RhoA and focal adhesions via NOX4-derived ROS activating associated RhoGEFs [247], perhaps via ROS-induced tyrosine phosphorylation. CAMK-II also promoted

migration via Rac [533]. As well as responding to inflammatory mediators [534], VSM NOX-derived ROS also positively influence vascular inflammation in hypertension. One study suggests that vascular oxidant stress is sensed by resident dendritic cells through the formation of isoketal protein adducts, leading to T-cell recruitment and activation and the release of cytokines that promote fibrosis and arterial stiffening [535].

FAK is a common point for cross-talk between GPCRs, RTKs and integrins. The 'inside-out' FAK activation pathway involves GPCR mediated NOX1 or NOX2-dependent activation of FAK, probably via Src [314], subsequent dissociation of ILK from clustered integrins, paxillin phosphorylation and RhoA activation, promoting migration through actin polymerisation and focal adhesion turnover at the leading edge [107, 529, 536-538]. There may then be secondary further integrin-dependent activation of NOX, possibly NOX4, following GPCR-induced FAK-activation, involving integrin $\alpha 1\beta 1$ [107]. FAK may also promote VSM proliferation and migration in response to enhanced perfusion pressure, because it will be activated by 'outside-in' stretch-induced integrin engagement [539]. FAK is also coupled to NOX4/Poldip2 activation and in combination they are capable of subsequently activating both Src and RhoA [49, 226, 229, 296, 539-541] and acts as an alternative platform for GRB2 docking and Ras/Raf/MEK/ERK signalling and PI3K/AKT activation, thus promoting both proliferation and migration of VSMCs in response to elevated pressure [107, 529, 539, 541]. Activation of FAK by stretch may also be ROS-dependent considering that NOX4 is located at focal attachments and potentially influences integrin-dependent signalling by oxidising integrin $\alpha 7\beta 1$ [49, 388]. FAK activation by NOX4 at focal adhesions probably involves oxidative inhibition of FAK phosphatases LMW-PTP or SHP2 [189, 542, 543]. Phosphorylation of Src, FAK and PYK2 are all increased in VSM of SHR [485, 544], while Ang II-induced ERK phosphorylation and impairment of vascular function are normalised by Src inhibition [101, 544].

In VSMCs, exogenous H_2O_2 induces transactivation of EGFR, PDGFR or IGFR, GRB2/Ras/Raf assembly and subsequent ERK phosphorylation [103, 173, 267, 274, 309, 545] and AKT activation [309]. Phosphorylation of EGFR, PDGFR and IGFR are also all enhanced in VSMC of SHR [485]. Evidence supports a role for Src or Fyn, PKC-δ, PYK2 and JAK2 in mediating these effects [173, 527, 546] and Src auto-phosphorylation is also enhanced in VSMC of SHR [485]. Knocking out SOD has a similar effect to that of exogenous ROS, promoting ERK and JAK/STAT phosphorylation and proliferation [547]. In addition to the proliferative, hypertrophic and migratory pathways mediated by ERK, JAK/STAT, PI3K/AKT and FAK/RhoA another important element of VSM hypertensive remodelling is nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). NF-κB is normally involved in inflammatory and cell survival responses to cellular stress such as cytokines and ROS [255, 534, 548] but becomes over-activated under oxidative stress, and contributes to the stimulation of VSM proliferation. NF-κB is activated by these stimuli through the degradation of an inhibitory I-κB protein and Src may contribute to ROS-dependent NF-κB activation in VSM by phosphorylating I-κB alpha [259, 549]. Alternatively, NF-κB may be activated via p38 MAP-kinase downstream of TNF-α receptor-1 stimulation via TRAF2 and ASK-1 [252, 550]. The NF-κB signalling pathway is opposed by nuclear factor erythroid 2-related factor 2 (Nrf2) which plays a cyto-protective role under oxidative stress by

inducing expression of anti-oxidant enzymes [548]. Nrf2 translocation to the nucleus is promoted by oxidative inhibition of KEAP-1 which degrades cytosolic Nrf2 [551].

In vivo, one would expect an early key response to oxidative stress to be excessive oxidative inhibition of PTPs and over-stimulation of NRTKs resulting in an overall enhancement of tyrosine phosphorylation, which would have consequences for both contractility and VSM growth/proliferation and migration [21]. One recent study suggests that coupled with ER stress, oxidative stress may induce VSM proliferation through irreversible hyper-oxidation (sulfinic and sulfonic acid) of PTPs [70]. Antioxidants also suppress many of the above described signalling pathways and processes associated with hypertension, including NRTK phosphorylation [110, 538, 546], growth factor receptor transactivation [103, 121, 136, 147, 149, 194, 274], AKT activation [109, 136, 150], ERK phosphorylation [64, 103, 109, 110, 136, 147, 149, 156, 194, 267, 274] and VSM hypertrophy [96, 552], proliferation [118, 121, 187, 267, 317, 485] and migration [100, 222, 229, 267, 538], in response to a range of GPCR agonists, growth factors and stretch (Table 4, Figures 7 & 8). A few studies highlight the importance of Src in hypertension, both as an upstream regulator of NOX and as a proximal ROS-sensitive kinase important in contraction and proliferation, by showing that oxidative stress and impaired cardiovascular function induced by Ang II infusion or in SHR vs. WKY rats are all improved by treatment with a Src inhibitor [82, 101, 485].

In animal models of hypertension, or in VSMC treated with hypertensive agents, the broad spectrum NOX/flavoprotein inhibitors diphenyleneiodonium (DPI) and apocynin inhibit growth-factor transactivation [103, 121, 136, 147, 149, 194, 316], ERK phosphorylation [64, 103, 109, 110, 121, 136, 147, 149, 156, 194, 316], AKT activation [109, 126], proliferation [79, 80, 107, 118, 121, 126, 127], hypertrophy [79, 80, 96, 109, 136, 147] and migration [100, 147, 178, 222]. Apocynin also normalises blood pressure and vasoconstrictor responses in spontaneously hypertensive rats [553, 554], DOCA salt rat [555] and dopamine D2 deficient mice [556]. A more recently developed inhibitor, VAS2870 also normalises MAP and improves acetylcholine-induced relaxation in isolated aorta [415]. While originally thought to be NOX-specific, DPI, apocynin and VAS2870 are considered as broad spectrum flavoprotein inhibitors, potentially acting on other sources of ROS or as direct antioxidants [474]. However, a new class of dual specific NOX inhibitors has been developed, including the NOX1/4 dual inhibitor GKT137831. This may be effective against hypertension induced intermittent hypoxia [557] and prevents hypertensive cardiac remodelling in rats [558], but does not significantly lower MAP in rats with hypertension induced by aortic banding [558]. GKT137831 has been deemed safe for human use and has progressed to clinical trials as a NOX blocker, but not yet for hypertension [474, 559]. Thus, the role for specific NOX isoforms in oxidative stress and its effects on the vascular wall in hypertension remains an avenue of active research.

6.4. Effects of selective NOX inhibition or over-expression in experimental hypertension. Several studies have examined the effects of knockout, siRNA depletion or over-expression of NOX isoforms or regulatory subunits on blood pressure, associated functional abnormalities and signalling in various mouse or rat models of hypertension. Ang II-induced hypertension and associated increases in ROS

production, ERK phosphorylation, proliferation and migration are blunted in $p47^{phox-/-}$ mice [41, 72, 72, 104, 166] or by $p22^{phox}$ siRNA or antisense [61, 157, 560], while the hypertrophic response is enhanced by over-expressing $p22^{phox}$ [552]. $p22^{phox-/-}$ or $p22^{phox}$ siRNA also reduce MAP in salt-loaded stroke-prone SHR [561] or Ang II-induced hypertension [61, 157]. Individually, there are multiple, but often conflicting reports over which NOX isoforms, if any, predominate in experimental hypertension and associated signalling.

There is disagreement over whether or not NOX1 contributes to baseline MAP in Ang II-infused mice [423, 490] and disagreement over whether or not the sustained phase of Ang II-induced hypertension is suppressed in $NOX1^{-/-}$ mice, with at least two saying it is [423, 490], and at least two saying it isn't [277, 562]. However, one study suggests that over-expressing NOX1 specifically in the VSM enhances Ang II-induced hypertension [520]. The Ang II pressor response in SHR is also blunted by NOX1 siRNA [483]. *In vitro*, the impaired Ach-induced relaxation in arteries from Ang II-induced hypertensive mice is restored by knocking out NOX1 [423]. There is also disagreement over whether $NOX1^{-/-}$ suppresses *in vivo* aortic wall thickening in Ang II-induced hypertension, one showing a reduction [490] and one showing no effect [423]. While *in vitro*, Ang II-induced aortic VSMC proliferation is inhibited by NOX1 siRNA [107], GPCR-induced transactivation of EGFR in human and mouse VSMCs is NOX1-dependent [125, 178], Ang II-induced aortic VSM hypertrophy is inhibited by NOX1 siRNA [89] and over-expressing NOX1 enhances this Ang II-induced hypertrophy [520]. There is some evidence that NOX1 is important in VSM migration. The increased activation of Src and FAK in kidneys of renal hypertensive mice is prevented by knocking out NOX1 [277], migration of human coronary artery VSMCs is NOX1-dependent [178] and Ang II-induced aortic VSMC FAK phosphorylation, ILK activation and migration are all inhibited by NOX1 siRNA [107].

There is also disagreement over whether NOX2 is an essential mediator of Ang II-induced hypertension in mice. Several studies suggest that NOX2 knockout lowers basal MAP and the acute phase of Ang II-induced hypertension, but not the sustained increase in MAP [277, 562, 563], while others show that the NOX2 inhibitor gp91ds-tat effectively suppresses Ang II-induced hypertension [98, 133]. In SHR, the acute Ang II-induced pressor response and myogenic contraction responses in renal afferent arterioles are blunted by NOX2 siRNA [483, 564]. There is also some evidence that NOX2-derived ROS negatively regulates endothelium-dependent relaxation. Ach-induced vasodilation is impaired in isolated aorta from mice with renovascular hypertension but not in $NOX2^{-/-}$ mice or in aorta from hypertensive mice treated with gp91ds-tat [425], while over-expressing endothelial NOX2 in mice potentiates the hypertensive effects of Ang II infusion without changing basal MAP [565]. An inhibitory role for NOX2 in the control of endothelium-dependent relaxation is supported by the observation that NO-dependent, flow-induced dilation is enhanced in humans with hereditary NOX2 deficiency [501]. NOX2 knockout also prevents Ang II infusion-induced aortic wall thickening [563] and NOX2 siRNA inhibits Ang II-induced aortic VSMC proliferation *in vitro* [107].

There are two opposing schools of thought about the role of NOX4 in the pathophysiology of hypertension, perhaps because it only generates H_2O_2 [45, 212-215], which is generally pro-relaxant

in the normotensive resistance vasculature, contributing to normal endothelial function [52, 209, 215, 291, 327, 422, 441, 444], but on the other hand its expression is upregulated in experimental hypertension [70, 423, 493] and by inflammatory mediators (eg TNF-α) in human aortic endothelium [263]. One theory is that like other NOX isoforms, NOX4 makes a direct contribution to oxidative stress and therefore to the pathogenesis of hypertension. Two studies show that $NOX4^{-/-}$ or $poldip2^{+/-}$ suppress Ang II infusion-induced increases in pulse pressure or MAP in mice [226, 566] while having no effect on baseline MAP or cardiac remodelling [566]. Yet another study suggests that NOX4 does contribute to pressure overload–induced cardiac dysfunction [264]. NOX4 may be pro-contractile in hypertension, as suggested by a study on the hypertensive effects of chronic intermittent hypoxia in rats. This was associated with an increased VSM expression of RhoA and Rho-kinase which was normalised by the selective NOX1/4 inhibitor GKT137831 [557]. Whether this increased expression of RhoA and Rho-kinase equates to an enhanced Ca^{2+} sensitizing contractile response to vasoconstrictor agents via MLCP inhibition, thus opposing the normal pro-relaxant effects of NOX4-derived H_2O_2, remains to be determined. A number of studies support a role for NOX4 in hypertensive vascular remodelling. Inhibiting NOX1/4 normalises mesenteric VSMC proliferation in SHR [70] and NOX4 appears to be the primary NOX isoform required for IGF-induced VSMC migration [222]. NOX4 may support proliferation via oxidative inhibition of SHP2 in association with PDGFR activation [258] and most likely supports VSMC migration in association with Poldip2, upstream of RhoA/FAK/integrin-mediated focal adhesion turnover and stress fiber formation [107, 228, 529, 536, 537].

The other theory is that upregulation of NOX4 in hypertension may be protective, particularly in the endothelium. Selectively over-expressing endothelial NOX4 reduces baseline MAP and prevents the initial transient phase of the hypertensive response to Ang II infusion but does not influence aortic wall thickness [215]. NOX4 is also upregulated by pathological oscillatory shear-stress or chronic flow overload in endothelium, but to a greater extent than is NOX2, thus potentially shifting the balance in ROS species away from pro-contractile superoxide towards pro-relaxant H_2O_2 [215, 327, 422, 444]. This is supported by the possible blood pressure lowering effect of VSM PKG activation by H_2O_2, probably derived from endothelial NOX4 [400]. In contrast to the endothelium-selective knockout described in [215], total NOX4 knockout has no effect on baseline MAP or the Ang II-infusion induced increase in MAP [52], but it enhances Ang II-induced aortic hypertrophy, impairs ACh-induced relaxation and potentiates Ang II induced expression of inflammatory cytokines, strongly suggesting a protective anti-hypertensive role for NOX4-derived H_2O_2 [52]. It is also suggested that NOX4-derived H_2O_2 is important in maintaining VSM differentiated state by promoting expression of contractile proteins and inhibiting proliferation, as opposed to NOX1 which is pro-proliferative [47, 567]. NOX4 may be anti-hypertensive and cardio-protective in part via induction of Nrf2 activity in VSM and heart [52, 568]. The idea of this protective NOX4/ Nrf2 axis is supported by the additional observation that prolonged superoxide generation by NOX1/2/5 (but not NOX4) generally results in activation of the pro-inflammatory and pro-proliferative transcription regulator NFκB [534, 548] (Figure 7). Importantly, if this protective effect of NOX4 holds true for human hypertension it may suggest potential detrimental side-effects or even exacerbation of symptoms with antioxidant therapy as a treatment for the disease.

NOX5 appears to be pro-proliferative in human VSMC and endothelium, acting via activation of MAP-kinases and JAK2 [57, 317]. However, a thorough analysis of a potential role for NOX5 in hypertension is hampered the fact that rodents do not possess this NOX isoform. A few recent studies have addressed this by examining the effects of artificially expressing human NOX5 in mice, often in specific cell types. Expressing NOX5 in mouse VSM enhances basal superoxide production, enhances U46619 and ET-1 induced contraction and suppresses ACh-mediated relaxation in isolated mesenteric artery, but curiously does not significantly affect mesenteric artery media/lumen ratio or vessel stiffness or Ang II infusion-induced hypertension [66]. Thus VSM NOX5 may be involved in the control of tissue blood flow but not in the control of MAP. However, a number of recent studies suggest that NOX5 may be important in human hypertension indirectly by contributing to kidney dysfunction. When expressed specifically in renal podocytes of mice it impairs glomerular barrier function and raises systolic blood pressure [569] and when expressed in mouse smooth muscle and mesangial cells it accelerates the development of diabetic nephropathy [570]. Thirdly, a study in human renal proximal tubule cells shows that in tubule cells from patients with essential hypertension NOX5 expression was several fold higher while NOX1, NOX2 and NOX4 were unaffected [497]. Furthermore, cells from hypertensive patients exhibited reduced sensitivity to dopaminergic agonists, correlating with impaired suppression of tubular ROS production and sodium transport, implicating renal NOX5-derived ROS in the pathogenesis of essential hypertension [497].

Although roles for individual NOX isoforms in specific aspects of hypertension pathology, such as endothelial dysfunction or VSM proliferation have been suggested, there remain incompatibly opposing views over which specific isoforms of NOX are most important in determining the resultant increased MAP. Considering the general consensus over the effectiveness of antioxidants and broad spectrum NOX inhibitors at lowering MAP, improving endothelial function and associated remodelling in various animal models, and bearing in mind that the same knockout mouse often produces opposing results in the same model of hypertension, it is difficult to explain the discrepancies. It may well be that NOX4 does indeed exert both protective and pathogenic effects in hypertension, but still there are discrepancies. One can only speculate about subtle differences in experimental approach, such as doses of Ang II, duration of infusion, or methods used to measure the various functional and anatomical changes. Results with NOX5 are fewer but more consistent, if mice expressing human NOX5 are to be taken as valid models of human disease.

7. NOX in the pulmonary circulation. As discussed in previous sections, in the systemic circulation as a whole we can make a number of generalisations about the role of NOX-derived ROS in the control of vascular tone: on the one hand, superoxide and H_2O_2 produced within the VSM contribute to agonist-induced contraction and myogenic tone, while on the other, ROS produced by the endothelium contribute to agonist or flow-induced relaxation, primarily mediated by endothelial H_2O_2. Functionally, the pulmonary circulation differs from the systemic circulation in that it must respond to increases in cardiac output with vasodilation, such as during exercise, to accommodate the increased flow. It is therefore a low resistance/low pressure system, exhibiting no flow-autoregulation and

minimal or absent myogenic contraction. While isolated arteries do constrict in response to prostaglandins, 5-HT, ET-1 and sphingolipids, the pulmonary vasculature operates largely independently of circulating vasoactive hormones and the autonomic nervous system, but does exhibit endothelium-dependent relaxation, largely mediated by NO·, but with an additional contribution of EHDF, which is relatively poorly characterised in this vascular bed [571-573]. Flow-induced dilation in the lung is believed to be achieved primarily through passive distension and recruitment of arterioles, although endothelium-dependent dilation may also contribute [291]. A perhaps unique feature of the pulmonary circulation is the fact that arteries constrict in response to hypoxia (hypoxic pulmonary vasoconstriction, HPV), as opposed to most systemic vessels, which dilate [574]. This contributes to ventilation/perfusion matching in healthy lungs, whereby regional hypoxia triggers constriction in that region, directing blood flow away from poorly ventilated areas to better ventilated areas of the lung, thus maximising blood oxygenation. This section will outline some of the key differences in the way pulmonary arteries respond to ROS, in comparison with systemic vessels, and the potential role of NOX in those differences.

7.1. Differences between pulmonary and systemic arteries. Within the context of this review, one may ask: are there fundamental differences in the balance between pro-contractile and pro-relaxant effects of NOX-derived ROS in pulmonary vs. systemic arteries? In terms of relaxation, very few studies have examined the role of ROS in endothelial function in pulmonary arteries and these appear to disagree on the key points. An earlier study, comparing isolated cat cerebral and pulmonary arteries, suggests that acetylcholine-induced relaxation is partly suppressed by blocking endothelial SOD in both vessel types but is suppressed by the addition of catalase in cerebral arteries only, suggesting an NO· scavenging contractile effect of superoxide in both vessels, but that a relaxant effect of endothelial H_2O_2 is present in cerebral but absent from pulmonary [437]. In contrast, two recent studies have suggested a role for endothelial H_2O_2 in mediating an EDHF response in the pulmonary circulation, one showing that shear-stress activates eNOS via endothelial NOX4-derived H_2O_2 [291] and another that bradykinin-induced relaxation is suppressed by catalase, making it H_2O_2-dependent [573].

As has already been noted in previous sections, the use of exogenous ROS is not a perfect tool for elucidating the various functions of endogenous ROS, but nevertheless, there are apparent differences in the way that pulmonary and systemic arteries respond to exogenously applied ROS, suggesting that perhaps the key differences between vascular beds lie in the effector pathways. The majority of studies in pulmonary arteries show that they constricts in response to exogenous H_2O_2, as well as to superoxide generated by X/XO or LY83583, whether it is applied in the presence of pre-constriction or not and regardless of concentration, whereas in systemic vessels, exogenous superoxide may have a mixed contraction/relaxation effect, depending on concentration, and H_2O_2 will usually relax pre-constricted systemic vessels, regardless of concentration [291, 573]. It is also worth reiterating that direct constriction induced by exogenous ROS, in the absence of any pre-constriction, is common in pulmonary but very rare in systemic vessels, particularly resistance sized vessels (Table 3). If effector pathways are different or responding to ROS differently in pulmonary arteries, what may these

differences be? When comparing the effects of exogenous superoxide on pulmonary and mesenteric arteries, a key difference is that the Rho-kinase signalling pathway responds to superoxide in pulmonary but not mesenteric arteries. This was illustrated by LY83583 inducing contraction in α-toxin permeabilised pulmonary but not mesenteric arteries and enhancing Rho-kinase dependent MYPT1 phosphorylation in pulmonary but not mesenteric arteries, even though GPCR-induced contraction is sensitive to Rho-kinase inhibition in both pulmonary and mesenteric arteries [351]. Alternatively, there may be major differences in the way ion channels, particularly K^+ channels, respond to ROS. These contractile signalling pathways and their influence by ROS in the pulmonary circulation are discussed further in relation to the HPV response.

7.2. Role for NOX-derived ROS in HPV. In the pulmonary artery, there is almost universal agreement that ROS are essential mediators of HPV, but there are a number of theories about how ROS production or the effects of ROS on VSM function during acute hypoxia could be different in pulmonary arteries compared to systemic, and some of these include a role for NOX in that difference. Initial studies suggested NOX as the main source of ROS during HPV [575, 576] and that both basal and stimulated NOX-derived ROS production is several fold higher in pulmonary artery VSM compared to coronary artery VSM, for example [67]. Other studies showed that while HPV is preserved in $gp91^{phox\ -/-}$ mice [577, 578] and unaffected by NOX2 siRNA [579], hypoxia induces translocation of $p47^{phox}$ in pulmonary VSM and acute hypoxia-induced H_2O_2 production and HPV are partially suppressed in $p47^{phox-/-}$ mice [165, 578] or by NOX4 siRNA [579]. Thus ROS derived from NOX4, with a possible contribution from NOX1, and none from NOX2, may be important in mediating HPV. Considering NOX as a possible ROS sensor in HPV, it was suggested that while levels of NOX4 expression in the two tissues were not different [67], higher expression of glucose-6-phosphate dehydrogenase in pulmonary compared to coronary VSM could support a greater availability of pentose phosphate pathway-derived NADPH and NADH during acute hypoxia, resulting in relatively greater NADPH- and NADH-dependent NOX-derived ROS production and downstream pro-contractile signalling [580]. However, it must now be acknowledged that the primary hypoxic sensor and leading source of any change in ROS during acute hypoxia almost certainly originates in the mitochondria. There are two key theories in this regard. The first theory is that acute hypoxia reduces the production of mitochondrial ROS, resulting in reduced $K_V1.5$ and/or $K_V2.1$ current amplitude, depolarisation and enhanced Ca^{2+} influx [581]. This theory is consistent with the widely observed activation of VSM K_V currents by ROS in various vascular beds (section 5.5), including pulmonary [351], but does not explain the apparent dependence of hypoxic pulmonary vasoconstriction on Rho-kinase mediated Ca^{2+} sensitization [271, 582, 583], RyR mediated Ca^{2+} release [375, 584, 585] and store-operated Ca^{2+} influx [368, 586]. The second, and perhaps now more widely accepted theory is that hypoxia *increases* the production of mitochondrial ROS [1, 2], permitting the also widely observed ROS-mediated activation of Rho-kinase, sarcoplasmic reticulum Ca^{2+} release and TRP channel mediated Ca^{2+} influx (sections 4.2-4.4). A recent study on the effects of cross-transplantation of mitochondria between pulmonary and femoral VSMCs supports this, showing that acute hypoxia increases ROS production in native VSMCs from both vessel types but does so to a greater extent in pulmonary and that mitochondrial cross-transplantation fundamentally reverses the differences between pulmonary and femoral ROS

production, $[Ca^{2+}]_i$ responses and contraction in response to hypoxia [584]. Nevertheless, the theory that hypoxia increases ROS production is also compatible with a secondary but potentially important role for NOX-derived ROS in HPV, using PKC as a link between the two sources of ROS. This was first suggested in perfused rabbit lungs where hypoxia appeared to stimulate PKC-dependent NOX activation [587]. It was then shown that PKC-ε in particular is activated by mitochondrial ROS during acute hypoxia in pulmonary VSM, but not in mesenteric, and that PKC-ε was required for HPV [588]. The connection with NOX was then made by showing that acute hypoxia enhanced $p47^{phox}$ dependent ROS production, presumably from NOX1, because NOX2 was not expressed, and that this ROS production and HPV were suppressed in PKC-$\varepsilon^{-/-}$ mice [165], indicating that PKC-ε activates NOX1 in response to mitochondrial ROS by phosphorylating $p47^{phox}$, as also described for PKC-δ and PKC-ζ (section 2.2). In contrast, this study also showed that hypoxia did not activate either PKC-ε or NOX in mesenteric arteries [165]. Interestingly, Src, another kinase known to be important in the assembly and activation of NOX1 or 2, is also activated by acute hypoxia via mitochondrial ROS [174]. There are as yet no reports of an involvement of NOX5 in HPV. As an additional effector pathway for ROS induced contraction during HPV, K^+ channel *inhibition* by hypoxia-induced, NOX4-derived ROS has been suggested [589], perhaps via ROS-induced channel degradation [590], as had been previously claimed for GPCR-induced contraction downstream of NOX-derived ROS in pulmonary artery [58, 146]. However, this is a relatively infrequently reported phenomenon in a wider context, with the majority of studies on K_V channels showing them to be activated by ROS, as discussed earlier in this review. It remains to be determined whether the inhibition of K_V currents by ROS is pulmonary-specific. A proposed model for the role of NOX in HPV is summarised in Figure 9.

7.3. Role of NOX in pulmonary hypertension. Pulmonary hypertension (PH) is defined as a mean pulmonary arterial pressure in excess of 25mmHg or in excess of 20 mmHg if there are other hemodynamic abnormalities present. PH has a number of causes, including idiopathic (genetic and pharmacologically induced, also known as pulmonary arterial hypertension, PAH)); and PH due to lung disease or chronic hypoxia [591]. There are other categories, but these two are the most widely studied, having approximate correlates in animal models. Common features of all types of PH are impaired endothelium-dependent relaxation, contractile hyper-responsiveness and vascular remodelling. As with systemic hypertension, oxidative stress is a central feature of the underlying pathophysiology of PH/PAH [59, 210, 234, 589, 592-599] and antioxidants or drugs that target specific sources of ROS, such as NOX, have been suggested as potential therapies for PH [600-604].

Research on enhanced pulmonary VSM contractility in PH has focussed on the mechanisms of elevated $[Ca^{2+}]_i$ and the role of RhoA/Rho-kinase, and as far as chronic hypoxia-induced PH is concerned many of the pathways implicated in normal physiological HPV, such as K_V channel inhibition, increased RyR-mediated Ca^{2+} release, increased LVGCC and TRPC-mediated Ca^{2+} influx and increased RhoA/Rho-kinase activity, are also implicated in PH [114, 605, 605-616], as well as NOX-dependent impairment of endothelium-dependent relaxation [594, 617]. Some of these studies suggest that many of the signalling proteins that respond to NOX-derived ROS in response to acute hypoxia are upregulated in chronic hypoxia-induced PH or monocrotaline (MCT)-induced PAH (a model of idiopathic PAH). These

include L-type ($Ca_V1.2$) and T-type (Ca_V 3.2) Ca^{2+} channels [610], RyR2 [611], TRPC1 and TRPC6 [612], Ca^{2+}-activated chloride channel (TMEM16A) [613] and RhoA and Rho-kinase [605]. In addition, in PASMC from patients with idiopathic PAH, expression of the Ca^{2+} sensing receptor is increased and this has been linked to increased Ca^{2+} influx via STIM/Orai/TRPC channels [614]. Conversely, expression of K^+ channels, including $K_V1.5$ and KCNK3 (TASK-1) are down-regulated by chronic hypoxia and by mutations in bone morphogenetic protein receptor-2 (BMPR2) a common cause of idiopathic PAH in humans [618-621]. Vascular remodelling in PH includes hyper-proliferation and migration of VSMC, endothelium and fibroblasts and muscularisation of normally non-muscular arterioles and, although oxidative stress is implicated in the pathogenesis of this, there appears to have been relatively little targeted research into the ROS-sensitive signalling pathways involved. What can be gleaned however is that the main pathways that operate in systemic hypertension, such as PTP oxidation [597], growth-factor transactivation [622], Nrf2 suppression [597], and kinases Src [623], PYK2 [624, 625], JAK2 [626], FAK [269], ERK [210, 525, 593, 625, 627] and PI3K/AKT [628, 629] all play major roles in PH/PAH. What may differ is the role of individual NOX isoforms and how their expression and activity is altered to promote remodelling and hyper-contractility. A number of pathways exist through which expression of NOX and associated signalling proteins may be altered in PH/PAH. These include hypoxia-inducible factor-1α (HIF-1α) because it is known to be activated by mitochondrial ROS [606, 612, 618, 619, 630, 631], the production of which is increased by hypoxia [1, 2]. Activation of HIF-1α by hypoxia may occur via the intermediary ROS-dependent activation of Src [632]. Also, as discussed further below, important roles for TSP-1 [633], NFκB [625, 633] and peroxisome proliferator-activated receptor gamma (PPARγ) [625, 633] in regulating NOX expression and associated pulmonary vascular remodelling have also been suggested.

Even though one study suggest that NOX1 is poorly expressed in PASMC of large pulmonary artery from human donors [210], NOX1 expression is several-fold higher in PASMC from patients with idiopathic PAH, particularly females and is further increased by treatment with either the estrogen metabolite 16α-hydroxyestrone (16α-HOE) or 5-HT, both of which have been associated with the pathogenesis of PAH in humans [59, 597, 634]. NOX1 expression is also upregulated by chronic hypoxia in human pulmonary artery fibroblasts [635]. Only a handful of studies have examined the role of NOX1 in animal models of PH/PAH, with occasional conflicting results. Chronic hypoxia induces pulmonary NOX1 expression in piglets [594, 617], but it is not altered in new-born lambs with PH induced by antenatal ligation of the ductus arteriosus [598]. In rats, chronic hypoxia appears not to alter NOX1 expression in pulmonary artery [589, 636] whereas MCT induces a 10-fold increase [593]. In the latter model, NOX1 siRNA normalises MCT-induced PASMC ERK phosphorylation, proliferation and migration [593]. NOX1 knockout in mice suppresses the increased right ventricular systolic pressure and prevents the right ventricular hypertrophy and vascular remodelling induced by chronic hypoxia [59]. $NOX1^{-/-}$ also suppresses oxidant stress and PASMC proliferation and reverses suppression of Nrf2 activity induced by 5-HT [597]. Curiously, another study shows the opposite of the above, wherein $NOX1^{-/-}$ *induces* PH under normoxic conditions [637].

NOX2 is also reportedly poorly expressed in PASMC of large pulmonary arteries from human donors [210], but unlike NOX1, PH in new-born lambs induced by antenatal ligation of the ductus arteriosus is associated with increased NOX2 expression [598]. In one study in rats, PH induced by both MCT and chronic hypoxia is associated with increased expression of NOX2 in pulmonary arterioles and in the endothelium, and it is suggested that this increase is due to a concomitant decrease in expression of caveolin-1 [234]. Alternatively, other studies in rats show that pulmonary NOX2 expression is not significantly influenced by chronic hypoxia [589, 596, 636] or MCT [593, 638], but that NOX2 activity may instead be increased [596]. Either an increased expression or an increased activity of NOX2 would be consistent with the inhibitory effect of gp91ds-tat on Ca^{2+} influx-independent contraction in pulmonary artery from chronic hypoxic rats [596]. It would also be consistent with the suppressing effect of NOX2 knockout on pulmonary constrictor hyper-responsiveness, PDGFR and AKT activation, vascular remodelling, increased right ventricular pressure and right heart hypertrophy induced by chronic hypoxia in mice [599, 639]. Another study in mice shows that chronic hypoxia does increase pulmonary NOX2 expression and suggests that this increase and the associated vascular remodelling are dependent on mitochondrial ROS [631].

In PASMC from idiopathic PAH patients, expression of NOX4 was increased 2.5 fold compared to matched controls [59, 629]. In cultured human PASMC, NOX4 expression is increased by chronic hypoxia and this is mediated in part by autocrine induction of IGF binding protein-3 [592, 635] or by upregulation of TSP-1 expression, increased NFκB activity and suppression of PPARγ (a negative regulator of proliferation) [625, 633]. In this instance, PYK2 appears to be upstream of NFκB activation and the downstream ERK phosphorylation required for proliferation [625]. NOX4 expression in human pulmonary artery is also increased by another key mediator of VSMC proliferation, transforming growth factor-β (TGF-β) [52, 210]. In new-born lambs with PH induced by ductus arteriosus ligation, NOX4 and $p22^{phox}$ expression is increased [598], and also in this model, cyclical stretch, which may exert a stronger influence on the vessel wall during PH, induces NOX4 expression via an increase in mitochondrial ROS production and induction of NFκB [640]. In rats, a number of studies show that pulmonary NOX4 expression is upregulated by chronic hypoxia [234, 589, 595, 629, 636], MCT [234, 595, 641], another model of spontaneous PH [595] and by human BMPR2 activation [642], while another study finds no significant effect of MCT on pulmonary NOX4 expression in rats [593]. Also in contrast to rat studies, in piglets there is apparently no effect of chronic hypoxia on pulmonary NOX4 expression [594]. In mice, pulmonary NOX4 expression is increased by chronic hypoxia [631, 643], chronic intermittent hypoxia (CIH) [639] and MCT [638]. In one of these studies, the enhanced NOX4 expression and the associated PASMC proliferation required hypoxic induction of HIF-1α [643], while in another, it and the associated vascular remodelling were also dependent on mitochondrial ROS, suggesting a link between mitochondrial ROS and cytosolic NOX4-derived ROS in PH [631], a parallel mechanism to that suggested for the acute HPV response [165, 588].

Of all the NOX isoforms expressed in the pulmonary vasculature, the greatest amount of work has been done on the role of NOX4 in the mechanisms of vascular remodelling associated with PH/PAH. Human PASMC proliferation induced by chronic hypoxia is suppressed by NOX4 siRNA [592, 627] and

the dual specific inhibitor GKT137831 [644]. GKT137831 also inhibits proliferation of these cells stimulated by the estrogen metabolite 16α–OHE and does so more effectively in PASMC from PAH patients [59]. NOX4 siRNA also inhibits proliferation induced by TGF-β in human PASMC [210, 592], by cyclical stretch in PASMC from new-born lambs with PH induced by ductus arteriosus ligation [598, 640] and by thrombin and human BMP2 in rat PASMC [642, 645]. Putative NOX4-specific inhibitors VCC588646 and VCC202273 also partially prevent pulmonary vascular remodelling induced by MCT in rat lung [595]. Interestingly, in this last study, pulmonary NOX4 was found to be expressed primarily in adventitial fibroblasts and there is emerging evidence for its contribute to PH through promoting fibrosis and arterial stiffening [595, 646]. In terms of downstream signalling, NOX4 contributes to the enhanced mTOR, S6K and AKT phosphorylation, cell survival and proliferation observed in PASMC from PAH patients [629], and it appears to contribute to human PASMC proliferation in response to chronic hypoxia in part via suppression of PPARγ expression, suggesting a reciprocal negative relationship between NOX4 and PPARγ [627]. In rat PASMC, NOX4 appears to act via protein stabilization of HIF-2α and induction of plasminogen activator inhibitor-1 [645], while in PASMC from new-born lambs with PH induced by ductus arteriosus ligation it acts via NFκB activation [598]. In models of PH/PAH, there appears to be no evidence for NOX4 contributing to pulmonary VSMC migration, although one may expect this based on its known association with integrins, FAK/ILK and migratory stimuli in systemic hypertension [107]. There is also evidence that NOX4 participates in aberrant elevated $[Ca^{2+}]_i$ in PH by suppressing PASMC $K_V1.5$ current [589] and by enhancing expression of TRPC1 and TRPC6 [642]. In stark contrast to the above, two studies in mice show little involvement of NOX4 in PH, despite evidence for NOX4 upregulation by chronic hypoxia [631, 643], one showing that $NOX4^{-/-}$ only reduces the chronic hypoxia-induced increase in right ventricular systolic pressure but has no effect on vascular remodelling [59], and the other showing no effect of $NOX4^{-/-}$ on chronic hypoxia-induced increases in pulmonary artery pressure or vascular remodelling [647].

As with systemic hypertension, this data provides conflicting evidence over the importance of NOX to PH/PAH, particularly to the associated vascular remodelling. The data possibly suggests that there are species-dependent differences over which NOX isoform may be most important in PH, perhaps NOX4 and NOX1 in human and rat, NOX1 and NOX2 in mice, with NOX2 appearing of minimal importance in rats and humans and NOX4 possibly of minimal importance in mice. However, evidence also conflicts between studies using the same model in the same species, so differences may be explained by the use of different output measures, time points and drug delivery methods or different approaches to data collection. Interestingly, there is very little evidence of a protective effect of NOX4 in PH, unlike in systemic hypertension. This is in line with NOX4 being purely pro-contractile in the healthy pulmonary circulation and primarily pro-relaxant in the systemic. However, one recent study raises doubts about this too, suggesting that H_2O_2, probably derived (although not proven) from NOX4, exerts a protective effect in parallel with the damaging effects described above [648]. This study presented evidence for an enhanced oxidative activation of PKGIα in lung tissues from humans with PH and an animal model of chronic hypoxia, and that this enhanced activity of PKGIα was protective via both enhanced VSM relaxation and suppression of vascular remodelling. Clearly more work needs to be done to clarify the importance of NOX to the pathophysiology of PH and PAH and if possible

determine whether there are specific NOX isoforms that may be most suitable as potential therapeutic targets. The potential role of NOX in the pathogenesis of PH/PAH is summarised in Figure 10.

8. Summary and future directions.

There exists a highly complex network of tightly regulated signalling pathways both upstream and downstream of NOX-derived ROS production that operates in subtly different ways for different NOX isoforms in the VSM and endothelium of different vascular beds. They respond to a variety of hormonal, chemical and mechanical stimuli, to produce a range of physiological responses in the healthy vasculature. This includes VSM contraction, endothelium-dependent relaxation, VSM gene expression, growth, proliferation and migration. Although much has been done to characterise the roles of specific NOX isoforms in these functions, more work is needed to clarify the importance of compartmentalisation, particularly of NOX4 and NOX5 which are both expressed in sarcoplasmic reticulum and NOX4 in mitochondria and nucleus. There is extensive but inconclusive, and occasional contradictory evidence that expression and activity of NOX isoforms and associated signalling pathways are altered in various animal models of hypertension, pulmonary hypertension and pulmonary arterial hypertension. A role for NOX-derived ROS in the vascular inflammation that underlies many cardiovascular diseases, as well as the role of adventitial NOX, requires further investigation. For appropriate therapies to be devised and tested for use in humans, further work is needed to clarify the relative importance of specific NOX isoforms in these diseases, particularly NOX4 which appears to exert both detrimental and protective effects in hypertension and possibly in PH/PAH as well. Thus, whether general antioxidants or non-selective and highly selective NOX inhibitors prove beneficial in the treatment of hypertension in humans may depend on the relative effects of suppressing pathological and protective ROS function as well as their contributions to normal vascular homeostasis.

Figure 1

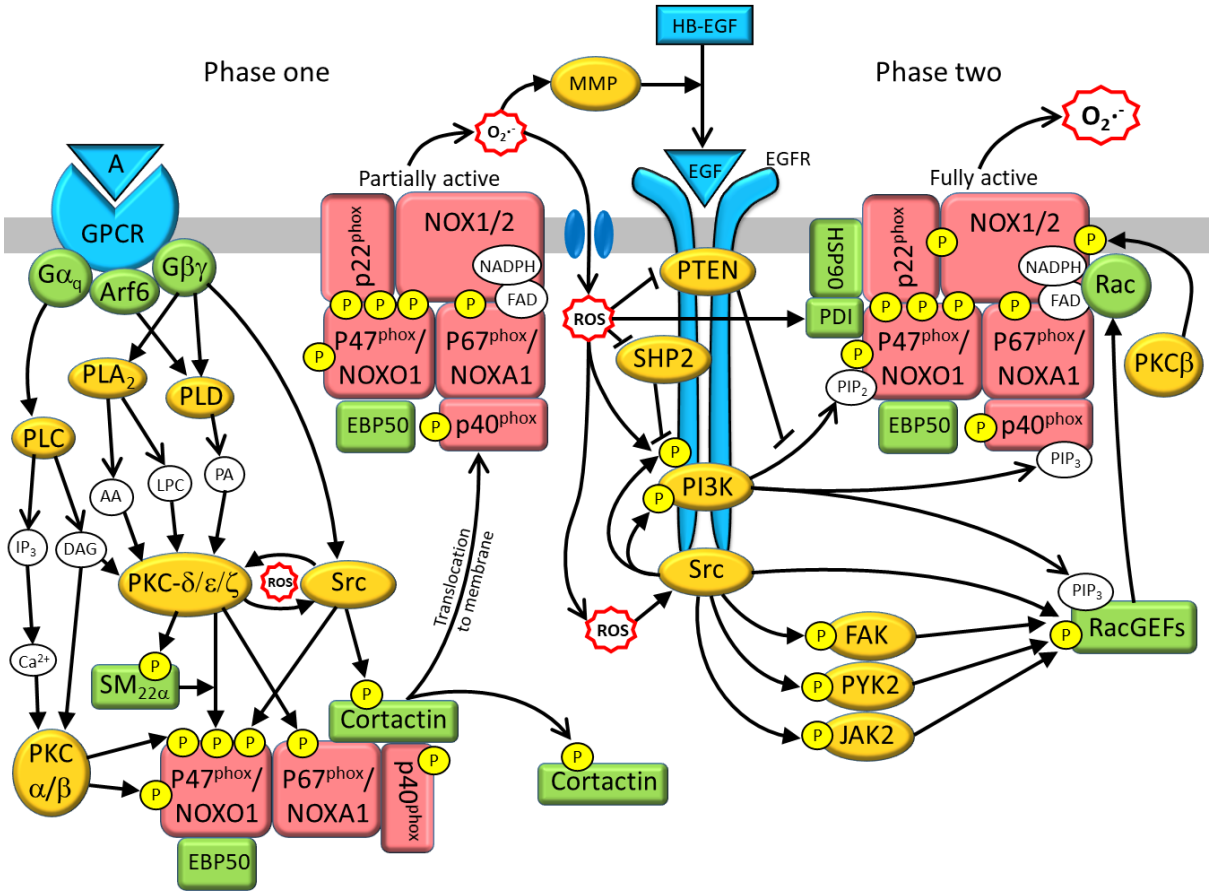
Phase one
Phase two
HB-EGF
MMP
EGF
EGFR
Partially active
Fully active
A
GPCR
Gαq
Gβγ
Arf6
PLA2
PLD
PLC
AA
LPC
PA
IP3
DAG
Ca2+
PKC-δ/ε/ζ
ROS
Src
SM22α
PKC α/β
p22phox
NOX1/2
NADPH
FAD
P47phox/ NOXO1
P67phox/ NOXA1
p40phox
EBP50
Translocation to membrane
Cortactin
PTEN
SHP2
PI3K
FAK
PYK2
JAK2
HSP90
PDI
PIP2
PIP3
Rac
RacGEFs
PKCβ

Figure 1. Activation of vascular NADPH oxidase-1 and -2 (NOX1/2) by G-protein coupled receptor (GPCR) stimulation. Inactive NOX1/2 exist in cell membranes coupled with $p22^{phox}$. Their activation follows a complex series of steps which can be divided into two phases. **Phase one**: Partial assembly and partial activation of the enzyme complex require translocation of regulatory subunits $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$ from the cytosol to the membrane. This is achieved through phosphorylation of $p47^{phox}$, and possibly also of $p67^{phox}$ and $p40^{phox}$, by multiple protein kinase C (PKC) isozymes and Src. PKC-δ may first need to phosphorylate $SM_{22\alpha}$ before it can phosphorylate $p47^{phox}$. NOXO1 may substitute for $p47^{phox}$ and NOXA1 may substitute for $p67^{phox}$. Src also phosphorylates cortactin, releasing it from the regulatory subunits, thus also supporting translocation. Thirdly, translocation is also facilitated through interaction with ezrin-radixin-moesin binding phosphoprotein 50 (EBP50). PKC isozymes are activated by GPCR through multiple pathways. Gq activates phospholipase C (PLC) which makes inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 triggers Ca^{2+} release from the sarcoplasmic reticulum to activate PKC-α or -β. DAG also is required for activation of PKC-α, $-\beta$, $-\delta$ and -ε. G$\beta\gamma$ subunits activate phospholipase A_2 (PLA_2) and phospholipase D (PLD), while PLD also requires the small G-protein ARF6. The products of these, arachidonic acid (AA), lysoposphatidylcholine (LPC) or phosphatidic acid (PA), also activate PKC-δ, $-\varepsilon$ and -ζ. Src is activated directly by G$\beta\gamma$. Alternatively, PKC and Src are capable of activating each other by phosphorylation and are both activated by other sources of ROS. **Phase two**: Full, complete activation of the oxidase requires the binding of active Rac to $p67^{phox}$ and the catalytic subunit. Rac activation requires prior activation of guanine nucleotide exchange factors (RacGEFs) and these are activated by PIP_3 and tyrosine phosphorylation by Src, FAK, PYP2 or JAK2. PIP_3 is generated by phosphatidyl inositol-3 kinase (PI3K) which is primarily activated following transactivation of the epidermal growth-factor receptor (EGFR) by Src. Low level ROS produced by the partially active NOX are able to diffuse into the cytosol via anion channels or aquaporins and may be sufficient to further activate Src as well as inhibit the tyrosine phosphatase SHP2 (inactivates PI3K) and the lipid phosphatase PTEN (degrades PIP_3 and PIP_2). EGFR may also be activated by epidermal growth-factor (EGF) generated by a ROS-activated matrix metalloprotease. Finally, full activity of the oxidase is promoted by further phosphorylation by PKC-β, binding of PIP_2 and PIP_3 and stabilization by heat-shock protein 90 (HSP90) and protein disulphide isomerase (PDI). PDI may form an intermolecular disulphide bond with $p47^{phox}$. Open arrowheads indicate movement or production of a substance. Closed arrowheads indicate an effect or response.

Figure 2

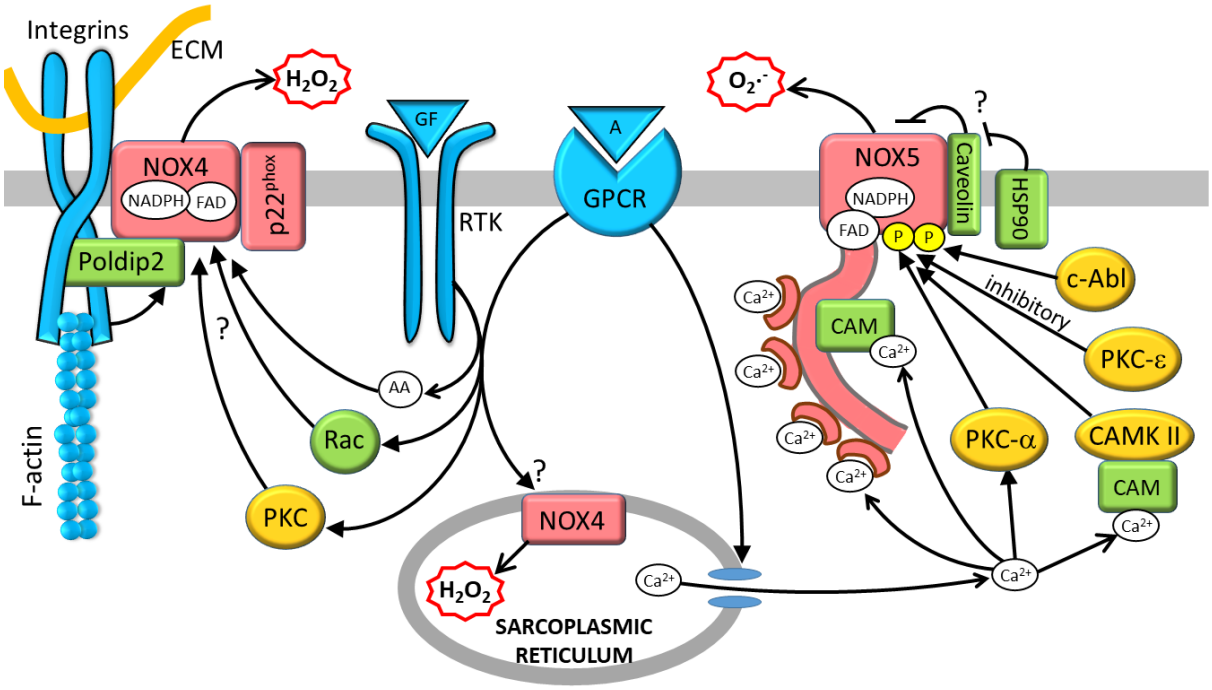

Figure 2. Activation of vascular NADPH oxidase-4 (NOX4) and -5 (NOX5) in vascular cells. NOX4 exists in cell membranes as a stable complex with $p22^{phox}$ and at the cell surface requires the additional binding of polymerase delta interacting protein 2 (Poldip2), which is associated with integrins and talin (not shown) at focal attachments in vascular smooth muscle. This may be sufficient for enzyme activity and H_2O_2 production, but activity may also be stimulated further by G-protein-coupled receptor (GPCR)-induced or receptor tyrosine kinase (RTK)-induced production of arachidonic acid (AA), activation of Rac or activation of protein kinase C (PKC). NOX4 is also present in the sarcoplasmic reticulum, although it is not clear whether or how it responds to cell surface receptor stimulation from this location or is simply constitutively active. NOX5 exists in cell membranes and, in endothelium, is associated with caveolin, which exerts an inhibitory effect on catalytic activity which in turn may be relieved by heat-shock protein 90 (HSP90). NOX5 is primarily activated acutely by an increase in intracellular Ca^{2+} concentration induced by GPCR, primarily via Ca^{2+} release from the sarcoplasmic reticulum (SR), which it senses via four 'EF hands' and Ca^{2+}-calmodulin (CaM) binding sites on a long intracellular auto-inhibitory domain. Sensitivity of NOX5 to Ca^{2+} may also be enhanced by phosphorylation of the catalytic subunit by PKC-α, Ca^{2+}-calmodulin-activated kinase II (CAMK-II) or c-Abl but may be inhibited by phosphorylation by PKC-ε. ECM = extracellular matrix. Open arrowheads indicate movement or production of a substance. Closed arrowheads indicate an effect or response.

Figure 3

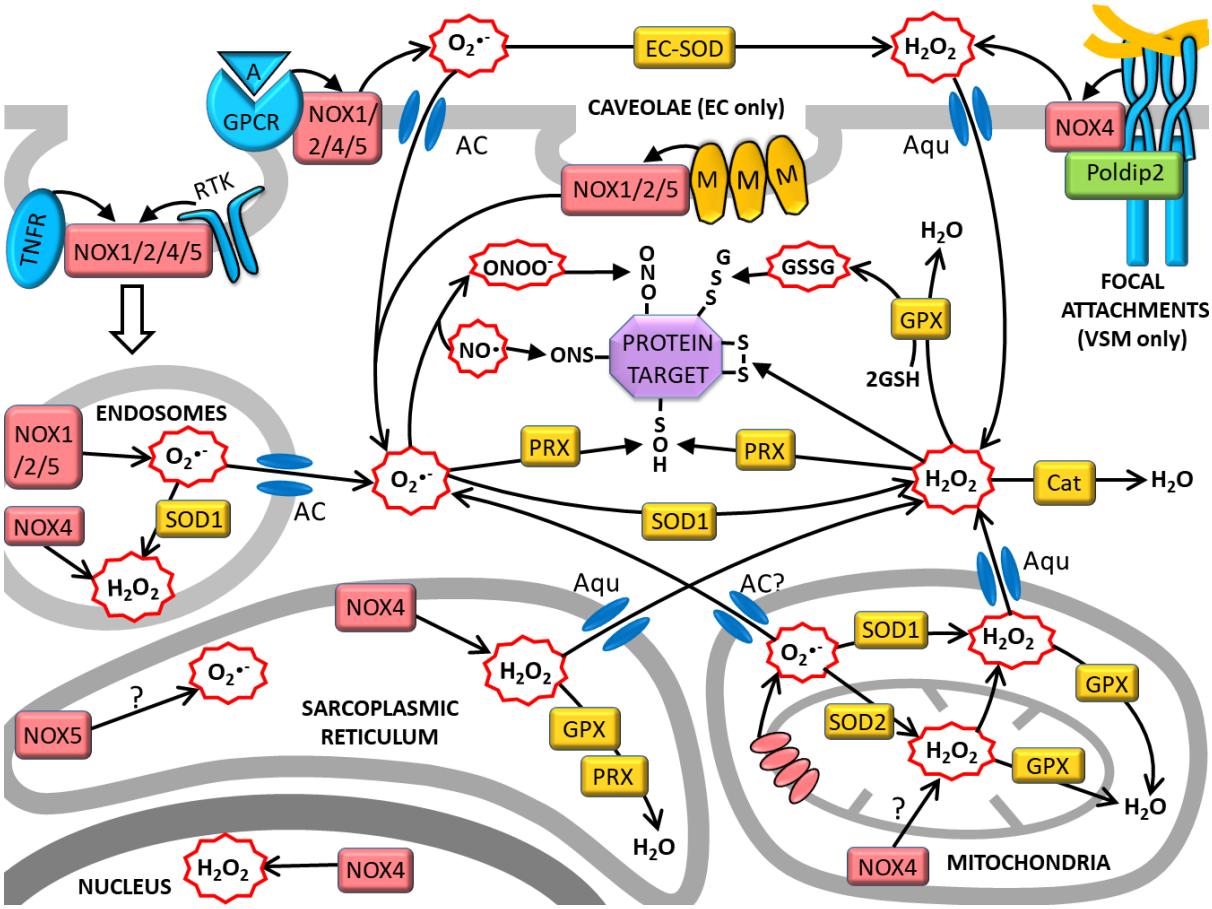
A
GPCR
NOX1/
2/4/5
$O_2^{\bullet-}$
EC-SOD
H_2O_2
CAVEOLAE (EC only)
AC
Aqu
NOX4
Poldip2
FOCAL
ATTACHMENTS
(VSM only)
RTK
TNFR
NOX1/2/4/5
NOX1/2/5
M
M
M
ONOO-
NO·
ONS
PROTEIN
TARGET
GSSG
GPX
H_2O
2GSH
ENDOSOMES
NOX1
/2/5
NOX4
SOD1
PRX
Cat
H_2O
AC
Aqu
AC?
NOX5
?
SARCOPLASMIC
RETICULUM
SOD2
MITOCHONDRIA
NUCLEUS

Figure 3. Compartmentalisation and metabolism of NADPH oxidase (NOX) isoforms. All NOX isoforms are expressed at the cell surface, but in endothelium NOX1, 2 and 5 are concentrated in caveolae (associated with the flow sensing 'mechanosome' (M)), while in vascular smooth muscle NOX4 is concentrated at focal attachments in association with polymerase delta interacting protein-2 (Poldip2). Superoxide produced at the cell surface in response to G-protein coupled receptor (GPCR) stimulation is extracellular, but can enter the cell through anion channels (AC, either chloride channel-3 or LRRC8) or be dismutated by extracellular superoxide dismutase (EC-SOD) into H_2O_2 which can enter the cell via aquaporins. Alternatively, all NOX isoforms may be endocytosed with activating receptors (eg. receptor tyrosine kinases (RTK) or TNF-α receptors (TNFR)). ROS are produced within the endosome to influence effector pathways there or can enter the cytosol through anion channels. NOX4 is highly expressed in the sarcoplasmic reticulum (SR) where it will influence protein folding and possibly Ca^{2+} handling. H_2O_2 is free to leave the SR via aquaporins. NOX5 is also expressed in the SR and NOX4 is also expressed in the mitochondria and nucleus. In the nucleus, NOX4 may contribute to the regulation of gene expression, while the function of NOX5 in the SR and NOX4 in the mitochondria is unknown. The main source of mitochondrial ROS is most likely complexes I and III of the electron transport chain. These are also free to enter the cytosol through aquaporins and possibly also anion channels. Superoxide and H_2O_2 oxidise cysteine (-SOH) and methionine (-SCHO, not shown) in target proteins, either directly or using oxidised PRX as an intermediate. Oxidation of adjacent cysteine residues may trigger formation of a disulphide bridge. Nitric oxide (NO·) modifies cysteine by nitrosylation (-SNO). Superoxide and NO· combine to form peroxynitrite ($ONOO^-$) which modifies proteins through nitration of tyrosine residues (-ONO). Glutathione peroxidase (GPX) uses the main intracellular reductant reduced glutathione (GSH) to reduce H_2O_2, producing oxidised glutathione (GSSG). GSSG may also modify proteins through glutathionylation (-SSG). Antioxidant enzymes are widely expressed in the cytosol and intracellular organelles where they will limit the accumulation of ROS and contribute to the compartmentalisation of ROS signalling. Superoxide is dismutated by SOD1 in the cytosol, endosomes and mitochondrial outer membrane and by SOD2 in the mitochondrial matrix. H_2O_2 is reduced by cytosolic catalase (Cat) and GPX, by GPX in the mitochondria and by GPX and PRX in the SR. GSSG is re-reduced by glutathione reductase, using NADPH (not shown). Open arrowheads indicate movement or production of a substance. Closed arrowheads indicate an effect or response.

Figure 4

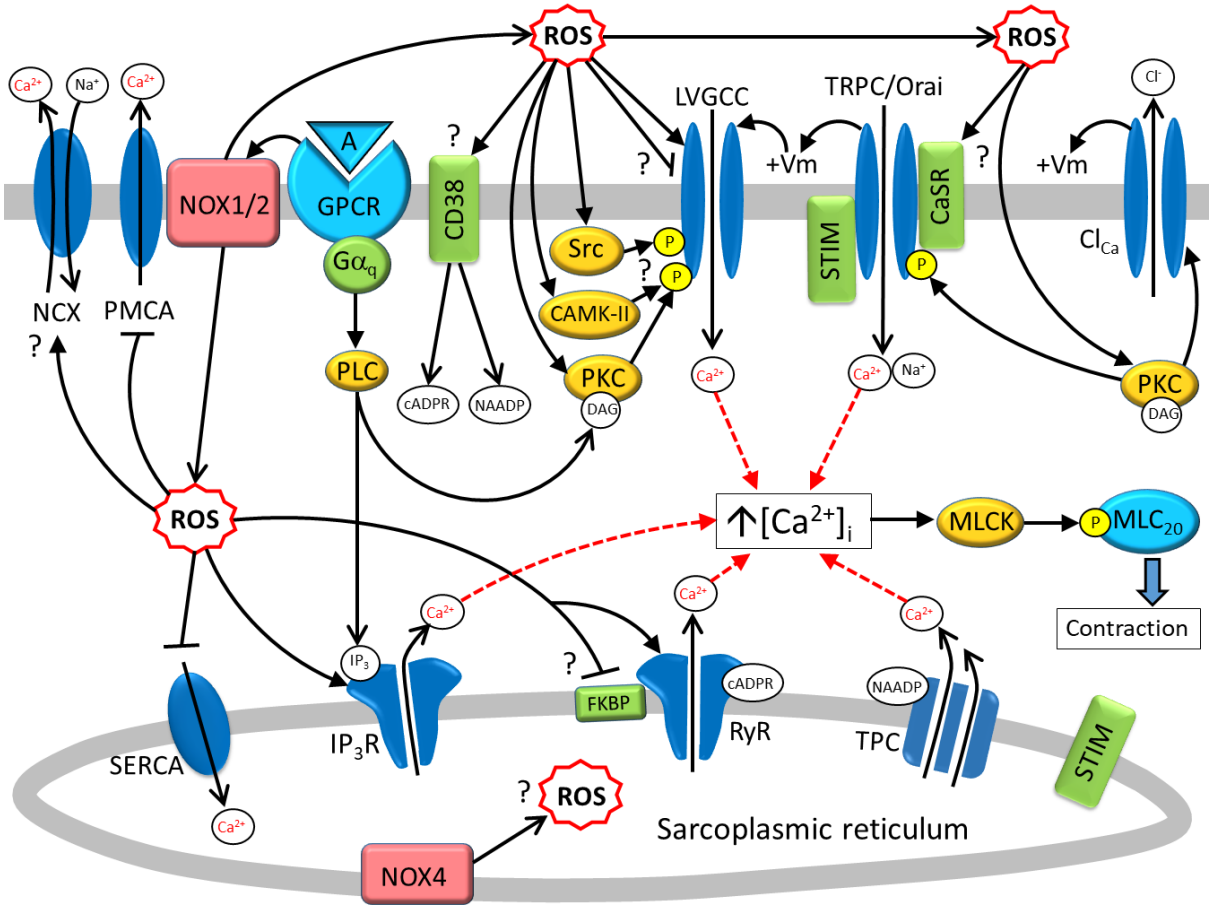

Figure 4. Role of NADPH oxidase (NOX) in vascular smooth muscle (VSM) Ca^{2+} signalling. G-protein coupled receptor (GPCR) stimulation activates phospholipase C (PLC, via G_q) and NOX1 or NOX2 on the cell surface. PLC generates inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 triggers Ca^{2+} release from the sarcoplasmic reticulum via IP_3R while DAG activates protein kinase C (PKC). NOX-derived ROS induce extracellular Ca^{2+} influx and Ca^{2+} release from the sarcoplasmic reticulum via multiple pathways. ROS stimulate Ca^{2+}-activated chloride channels (Cl_{Ca}) and TRPC1/6/Orai, the latter probably via stimulation of PKC-dependent phosphorylation. Opening of these channels depolarises the cell membrane, thus opening L-type voltage-gated Ca^{2+} channels (LVGCC). ROS may further enhance LVGCC opening through channel phosphorylation by Src, PKC or CAMK-II, or by direct cysteine oxidation, but whether the latter is stimulatory or inhibitory is unclear. ROS promote Ca^{2+} release from the sarcoplasmic reticulum by enhancing the sensitivity of IP_3R to IP_3 and by activating the ryanodine receptor (RyR). RyR is also activated by ROS indirectly by displacing the inhibitory protein FKBP from RyR and by stimulating CD38, which makes cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP). cADPR further stimulates RyR while NAADP stimulates Ca^{2+} release via the Two-Pore channel (TPC). ROS also help maintain an elevated $[Ca^{2+}]_i$ by inhibiting Ca^{2+} extrusion via the plasma membrane Ca^{2+} ATPase (PMCA) and re-uptake into the sarcoplasmic reticulum via the sarco-endoplasmic Ca^{2+} ATPase (SERCA). Finally, ROS also may stimulate the Na^+/Ca^{2+} exchanger, but this may oppose the other effects by extruding Ca^{2+}. NOX4 is expressed in the sarcoplasmic reticulum of VSM, but details of its contributions to Ca^{2+} homeostasis are unknown. Open arrowheads indicate movement or production of a substance. Closed arrowheads indicate an effect or response.

Figure 5

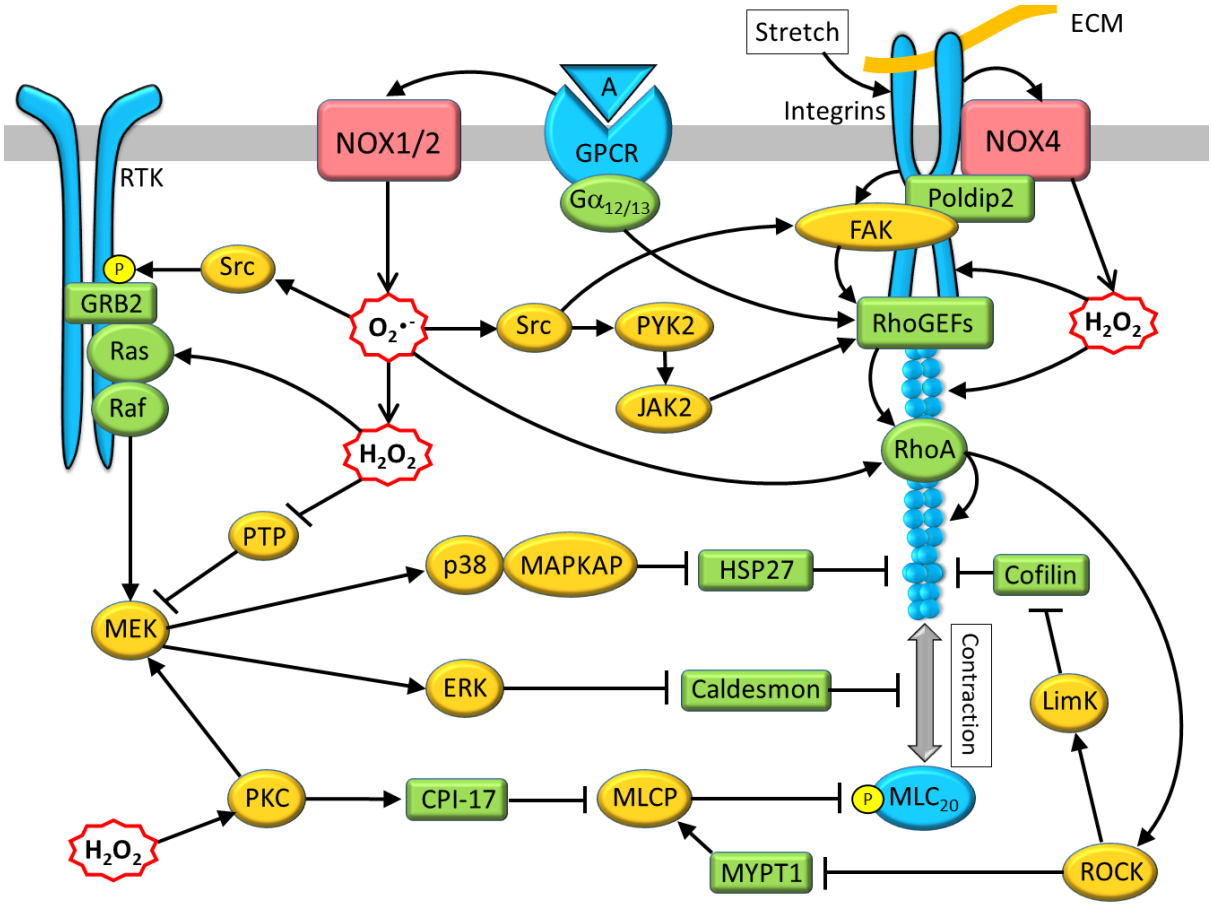
Stretch
ECM
Integrins
A
GPCR
Gα12/13
NOX1/2
NOX4
RTK
Poldip2
FAK
Src
GRB2
Ras
Raf
O2•-
Src
PYK2
JAK2
RhoGEFs
H2O2
H2O2
RhoA
PTP
p38
MAPKAP
HSP27
Cofilin
MEK
ERK
Caldesmon
Contraction
LimK
PKC
CPI-17
MLCP
MLC20
H2O2
MYPT1
ROCK

Figure 5. Role of NADPH oxidase (NOX) in Ca^{2+}-independent pro-contractile signalling in vascular smooth muscle. Contractile force may be enhanced without an increase in $[Ca^{2+}]_i$ through two main pathways: inhibition of myosin light-chain phosphatase (MLCP) and actin polymerisation. MLCP is negatively regulated by Rho-kinase (ROCK) through inhibitory phosphorylation of myosin phosphatase regulatory subunit-1 (MYPT1). ROCK is activated by RhoA. RhoA is either activated directly by superoxide or by Rho guanine nucleotide exchange factors (RhoGEFs). RhoGEFs are activated by G-protein coupled receptors (GPCR) either directly via $G_{12/13}$, or indirectly by NOX1- or NOX2-dependent tyrosine phosphorylation by Src, PYK2 or JAK2. RhoGEFs are also activated by stretch via integrin engagement with extracellular matrix (ECM) and subsequent activation of focal adhesion kinase (FAK), in association with H_2O_2 produced by NOX4 coupled to polymerase delta interacting protein-2 (Poldip2). RhoA promotes actin polymerisation either directly or indirectly by promoting ROCK and LIM kinase (LIMK)-induced inhibition of the actin severing protein cofilin. Additional routes for the promotion of actin polymerisation are by direct oxidation of actin by NOX4-derived H_2O_2, inhibition of the actin chaperone heat-shock protein 27 (HSP27) by p38 MAP-kinase and MAP-kinase activated protein kinase (MAPKAP) and ERK MAP-kinase-mediated inhibition of caldesmon which inhibits actin-myosin cross-bridge cycling. p38 and ERK MAP-kinases are both activation by ROS via MEK. NOX-derived H_2O_2 activates MEK via Src-dependent transactivation of receptor tyrosine kinases (RTK) and subsequent recruitment of growth-factor receptor binding protein-2 (GRB2), Ras and Raf, by direct oxidation of Ras, by oxidative inhibition of a MEK phosphatase or via protein kinase C (PKC) directed phosphorylation. PKC also inhibits MLCP by activating the inhibitory accessory protein C-kinase potentiated protein phosphatase-1 Inhibitor-17 (CPI-17). Open arrowheads indicate movement or production of a substance. Closed arrowheads indicate an effect or response.

Figure 6

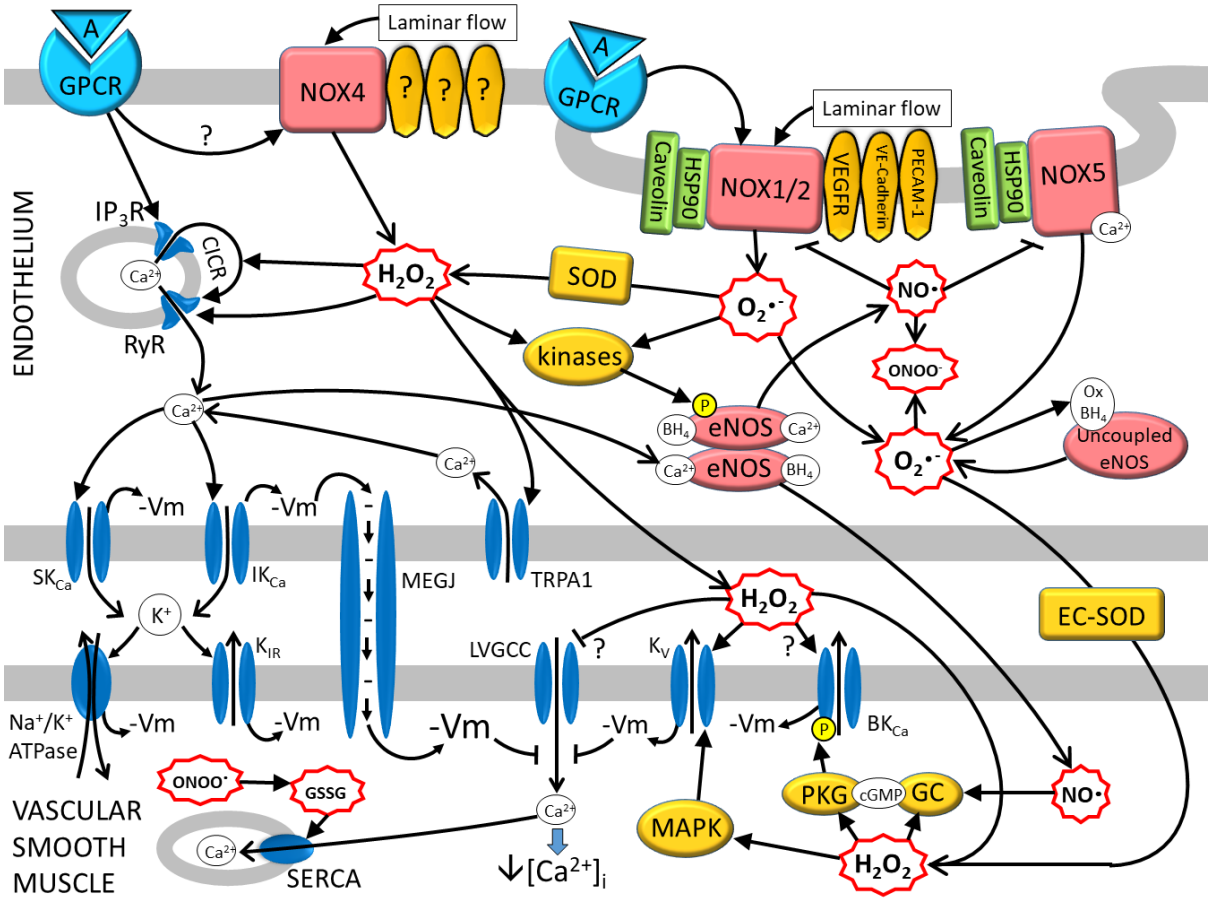

Figure 6. Role of NADPH oxidase (NOX) in endothelium-dependent relaxation of the vasculature. NOX1, 2, 4 and 5 are all expressed in the endothelium and 1, 2 and 5 are present in the caveolae. All isoforms are activated by pro-relaxant G-protein coupled receptor (GPCR) agonists and by flow via the endothelial flow 'mechanosome'. In caveolae, this consists of VEGFR, VE-cadherin and PECAM-1 and is associated with closure of K_{ATP} and opening of T-type Ca^{2+} channels (not shown). Caveolin-1 acts as a negative regulator of NOX1, 2 and 5 but this inhibition can be relieved by HSP90. The mechanism of NOX4 activation by GPCR and the exact nature of the NOX4 flow sensor are unclear. NOX4 generates H_2O_2 while NOX1/2/5 make superoxide, some of which is dismutated to H_2O_2 by SOD in the endothelium. GPCR also raise endothelial $[Ca^{2+}]_i$ by triggering release from the sarcoplasmic reticulum via IP_3R and RyR, and this Ca^{2+} stimulates eNOS to make NO·, which diffuses to the vascular smooth muscle (VSM) to activate guanylate cyclase (GC). GC makes cGMP which activates protein kinase G (PKG). PKG phosphorylates and activates large conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) to hyperpolarise the VSM, lower $[Ca^{2+}]_i$ and induce relaxation by closing L-type voltage-gated Ca^{2+} channels (LVGCC). Endothelial ROS, principally from NOX4, further promote eNOS activity by activating kinases which phosphorylate eNOS and by further raising endothelial $[Ca^{2+}]_i$ by enhancing Ca^{2+} release via RyR and Ca^{2+} influx via TRPA1 channels. VSM Endothelial Ca^{2+} (enhanced by H_2O_2) also activates the small and intermediate conductance Ca^{2+}-activated K^+ channels (SK_{Ca} and IK_{Ca}). The extruded K^+ diffuses to the VSM and activates the Na^+/K^+ATPase and inward rectifier (K_{IR}) channels to hyperpolarise the VSM, while endothelial hyperpolarisation may also spread to the VSM via myo-endothelial gap

junctions (MEGJ). NOX4-derived H_2O_2 and any dismutated superoxide from NOX1, 2 or 5 also acts as a direct hyperpolarising factor. It diffuses to the VSM where it directly activates BK_{Ca} and voltage-gated K^+ channels (K_V), further activates GC and PKG and activates MAPK to further stimulate K_V channels, thus further hyperpolarising the VSM. Superoxide derived from endothelial NOX1, 2 and 5 partly antagonises eNOS by oxidising BH_4, thus uncoupling eNOS, forcing it to make more superoxide. Conversely, NO· also limits superoxide production by inhibiting the activity of NOX1, 2 and 5. NO· and superoxide react to make $ONOO^-$. In the VSM, this promotes formation of oxidised glutathione (GSSG) which may activate SERCA, enhancing the rate of VSM Ca^{2+} uptake into the sarcoplasmic reticulum, thus promoting relaxation. H_2O_2 may also directly inhibit LVGCC. VEGFR = vascular endothelial growth-factor receptor, PECAM-1 = platelet-endothelial cell adhesion molecule-1, HSP90 = heat-shock protein 90, SOD = superoxide dismutase, EC-SOD = extracellular SOD, IP_3R = inositol trisphosphate receptor, RyR = ryanodine receptor channel. Open arrowheads indicate movement or production of a substance. Closed arrowheads indicate an effect or response.

Figure 7

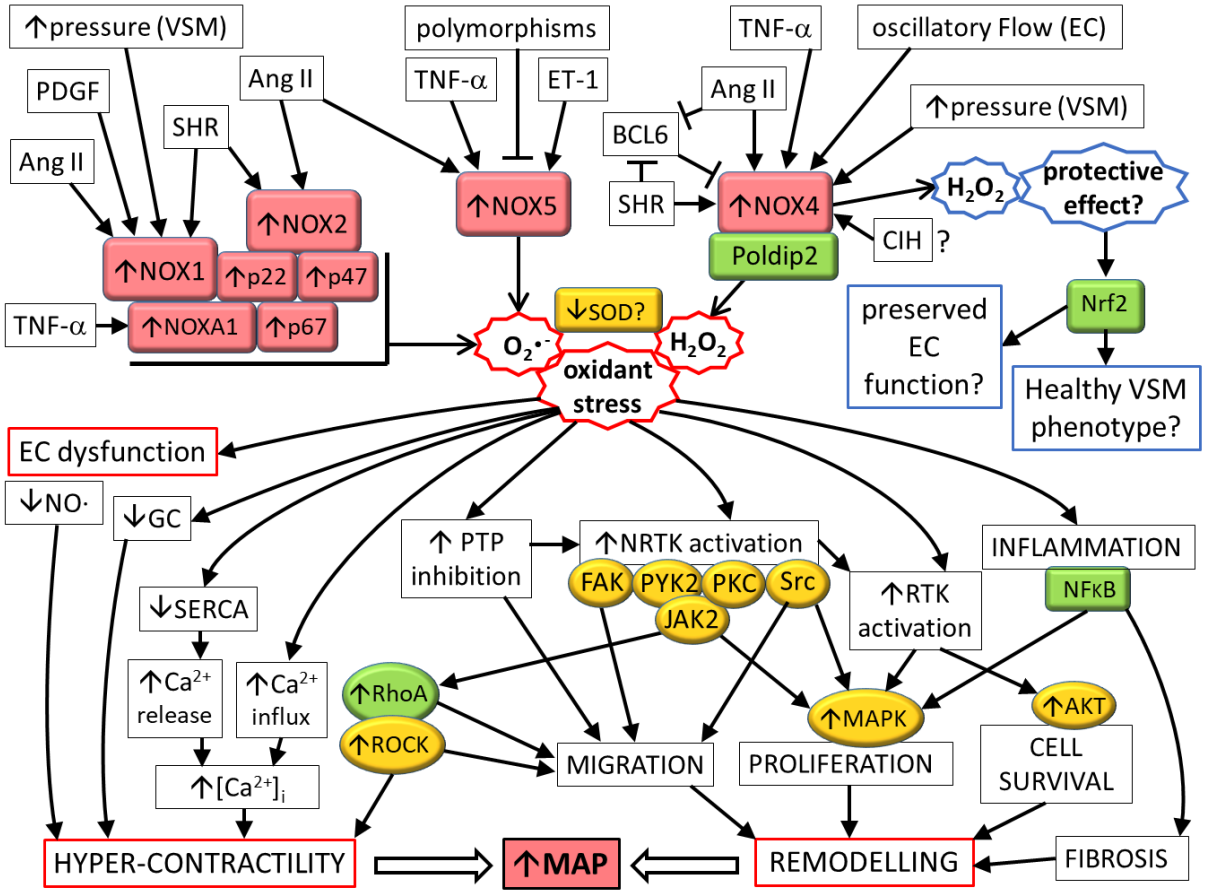
↑pressure (VSM)
PDGF
Ang II
SHR
Ang II
↑NOX2
↑NOX1
↑p22
↑p47
TNF-α
↑NOXA1
↑p67
polymorphisms
TNF-α
ET-1
↑NOX5
Ang II
TNF-α
oscillatory Flow (EC)
↑pressure (VSM)
BCL6
SHR
↑NOX4
Poldip2
H2O2
protective effect?
CIH
?
↓SOD?
O2•-
H2O2
oxidant stress
preserved EC function?
Nrf2
Healthy VSM phenotype?
EC dysfunction
↓NO·
↓GC
↓SERCA
↑Ca2+ release
↑Ca2+ influx
↑[Ca2+]i
↑ PTP inhibition
↑NRTK activation
FAK
PYK2
PKC
Src
JAK2
↑RTK activation
INFLAMMATION
NFκB
↑RhoA
↑ROCK
↑MAPK
↑AKT
MIGRATION
PROLIFERATION
CELL SURVIVAL
HYPER-CONTRACTILITY
↑MAP
REMODELLING
FIBROSIS

Figure 7. Proposed role of vascular NADPH oxidase (NOX) in the pathogenesis of hypertension. Multiple mitogenic and pro-contractile stimuli (Ang II, ET-1, PDGF, TNF-α) induce an increased vascular expression of NOX1, NOX2, regulatory subunits $p22^{phox}$ (p22), $p47^{phox}$ (p47), $p67^{phox}$ (p67) and NOXA1, and NOX5. Vascular NOX1 and NOX2 expression are also increased in the spontaneously hypertensive rat (SHR). NOX5 activity may be suppressed by polymorphisms in humans, while activity of $p22^{phox}$ may be increased (not shown). NOX4 expression is upregulated by inflammatory mediators (eg TNF-α) and mechanical forces associated with cardiovascular disease: oscillatory flow in endothelium (EC) and elevated perfusion pressure in vascular smooth muscle (VSM). Most studies agree that NOX4 expression is enhanced in SHR and in Ang II-induced hypertension, and this may be via inhibition of BCL6 expression, thus relieving BCL6-induced suppression of NOX4 expression. NOX4 expression may also be enhanced by chronic intermittent hypoxia (CIH). Collectively, the result of enhanced NOX expression is the production of excess superoxide ($O_2^{\bullet -}$, NOX1, NOX2 and NOX5) and H_2O_2 (NOX4), resulting in oxidant stress. Accumulation of superoxide may be exacerbated by reduced expression or activity of superoxide dismutase (SOD). Oxidant stress impairs endothelium-dependent relaxation through the combined effects of NO· scavenging and eNOS uncoupling. Excess superoxide also impairs guanylate cyclase (GC) activity. Vascular hyper-contractility is further exacerbated by elevated VSM $[Ca^{2+}]_i$, through suppression of SERCA activity, enhanced Ca^{2+} release from the sarcoplasmic reticulum and elevated Ca^{2+} channel activity, and through increased expression and activity of RhoA and Rho-kinase (ROCK). Vascular remodelling occurs through the combined action of ROS-stimulated VSMC migration (mediated by FAK, ILK, integrins and RhoA/ROCK), proliferation (primarily ERK MAP-kinase mediated, MAPK), increased survival (PI3K and AKT mediated) and fibrosis, coupled to inflammation. These processes are promoted by oxidative inhibition of protein tyrosine phosphatases (PTP) and enhanced activity of non-receptor tyrosine kinases (NRTK) and receptor tyrosine kinases (RTK). The NRTKs FAK, Src, PYK2 and JAK2 as well as protein kinase C (PKC) are all essential co-ordinators of vascular remodelling (see Figure 8 for more details). They also promote hyper-contractility via RhoA/ROCK. VSM proliferation and fibrosis is also supported by oxidative activation of the transcription regulator nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB). Opposing the 'oxidant stress' effects of enhanced NOX4 expression, excess H_2O_2 may also have a protective effect in hypertension, via activation of nuclear factor erythroid 2-related factor 2 (Nrf2), which may act to improve endothelial function and maintain a differentiated VSM phenotype. TNF-α = tumor necrosis factor-α, PDGF = platelet derived growth factor. Open arrowheads indicate movement or production of a substance. Closed arrowheads indicate an effect or response.

Figure 8

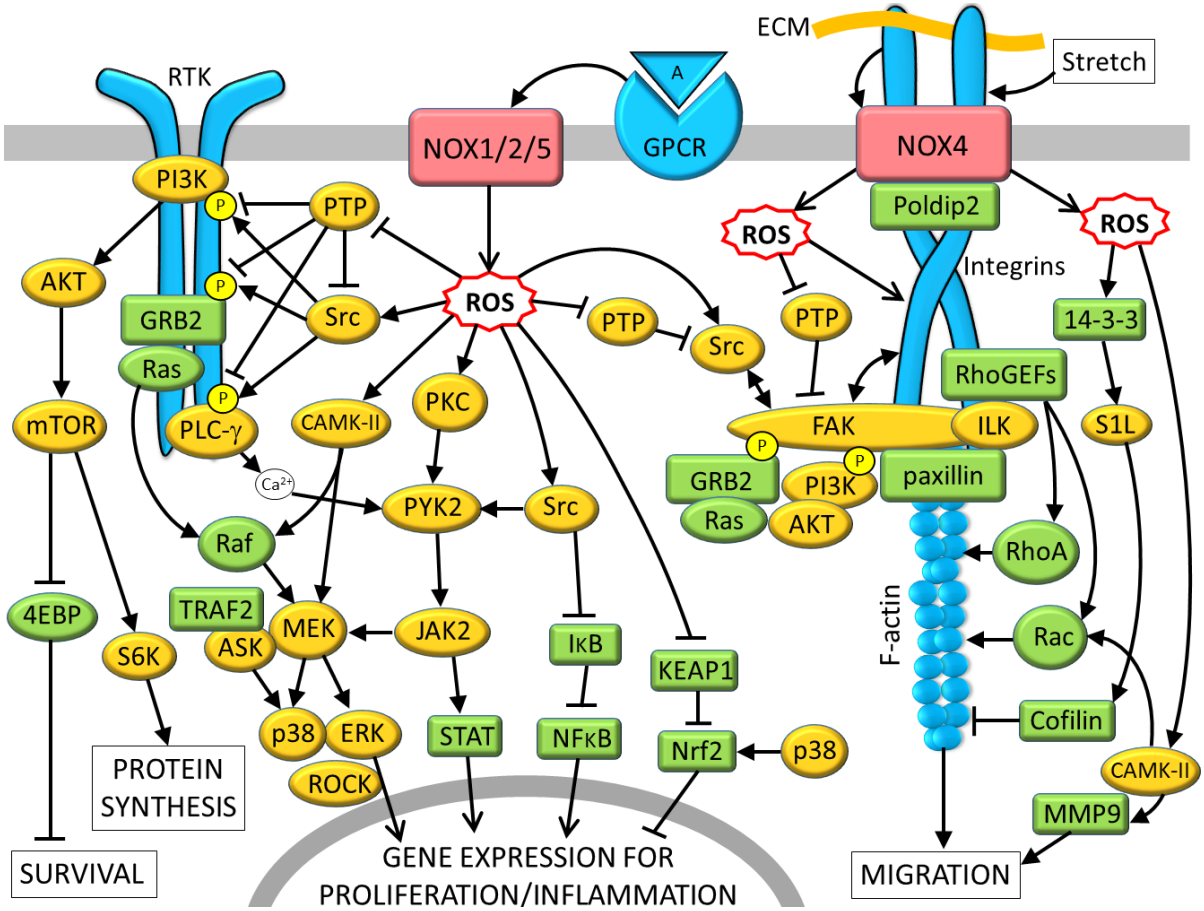
ECM
Stretch
RTK
A
GPCR
NOX1/2/5
NOX4
PI3K
P
PTP
ROS
Poldip2
ROS
AKT
Integrins
GRB2
Src
ROS
PTP
PTP
14-3-3
Ras
Src
RhoGEFs
mTOR
PLC-γ
CAMK-II
PKC
FAK
ILK
S1L
GRB2
PI3K
paxillin
Ca2+
PYK2
Src
Ras
AKT
Raf
RhoA
4EBP
TRAF2
MEK
JAK2
IκB
KEAP1
F-actin
Rac
S6K
ASK
Cofilin
p38
ERK
STAT
NFκB
Nrf2
p38
CAMK-II
PROTEIN SYNTHESIS
ROCK
MMP9
SURVIVAL
GENE EXPRESSION FOR PROLIFERATION/INFLAMMATION
MIGRATION

Figure 8. Role of NADPH oxidase (NOX) in vascular smooth muscle growth, proliferation and migration in response to G-protein coupled receptor activation and stretch. NOX1, 2 or 5 in the plasma membrane or endosomes are activated by G-protein coupled receptor (GPCR) agonists. The ROS generated produce parallel inhibition of multiple protein tyrosine phosphatases (PTPs), and activation of multiple non-receptor tyrosine kinases (Src, PYK2 and JAK2), CAMK-II and PKC. One consequence of this is Src-mediated transactivation of receptor tyrosine kinases (RTKs) which act as platforms for the parallel activation of PI3K, Ras and PLC-γ. PI3K activates AKT and mTOR and downstream protein synthesis and cell survival responses via S6K and 4EBP. Via GRB2, Ras activates Raf, MEK and ERK MAP-kinase (ERK) to promote cell division and proliferation. Alternatively, Raf and MEK activation occur via CAMK-II-mediated or PKC/PYK2/JAK2-mediated phosphorylation. Nuclear translocation of ERK is supported by Rho-kinase (ROCK). PLC-γ triggers Ca^{2+} release from the sarcoplasmic reticulum, which activates PYK2 and then JAK2. JAK2 also supports proliferation via STAT and ERK. In addition, Src promotes activity of the pro-proliferative transcription regulator NFκB via inhibition of IκB. p38 MAP-kinase (p38) is also involved in stimulating proliferation downstream of MEK or TRAF2/ASK, and contributes to the activation of NFκB, particularly in response to TNF-α receptor activation. Both p38 activation pathways are NOX-dependent (not shown). As a braking mechanism to this, ROS also promote activity of the cyto-protective transcriptional regulator Nrf2 via degradation of the inhibitory KEAP1. Via Src, GPCR-induced ROS may also activate FAK at focal adhesions. This may lead to 'inside-out' activation of integrins and activation of NOX4. Alternatively, stretch may activate integrins leading to 'outside-in' activation of FAK and NOX4. Either way, activated FAK also acts as a platform for the GRB2/Ras proliferation pathway and the PI3K/AKT growth and cell survival pathway, while FAK and NOX4-derived ROS will promote actin polymerisation/de-polymerisation, focal adhesion turnover and migration via ILK/paxillin and RhoA, Rac and cofilin activity. CAMK-II also promotes migration via Rac or MMP9. NOX4 in the mitochondria or nucleus and NOX4 and NOX5 in the sarcoplasmic reticulum may also contribute to these responses through as yet uncharacterised pathways (not shown). CAMK-II = Ca^{2+}-calmodulin activated kinase-II. PYK2 = protein tyrosine kinase-2β. JAK2 = Janus kinase-2. MMP9 = matrix metalloprotease-9. PKC = protein kinase C. PI3K = phosphatidyl inositol-3 kinase. mTOR = mammalian target of rapamycin. S6K = S6-kinase. 4EBP = eukaryotic translation initiation factor 4E-binding protein. GRB2 = growth-factor receptor bound protein-2. PLC-γ = phospholipase C-γ. STAT = signal transducer and activator of transcription. NFκB = nuclear factor kappa-light-chain-enhancer of activated B cells. ASK = apoptosis signal-regulating kinase 1. IκB = inhibitor of κB. Nrf2 = nuclear factor erythroid 2-related factor 2. Poldip2 = polymerase delta interacting protein-2. FAK = focal adhesion kinase. ILK = integrin-linked kinase. RhoGEFs = Rho guanine nucleotide exchange factors. S1L = Slingshot-1L. Double-headed arrows indicate a bi-directional activation pathway. Open arrowheads indicate movement or production of a substance. Closed arrowheads indicate an effect or response.

Figure 9

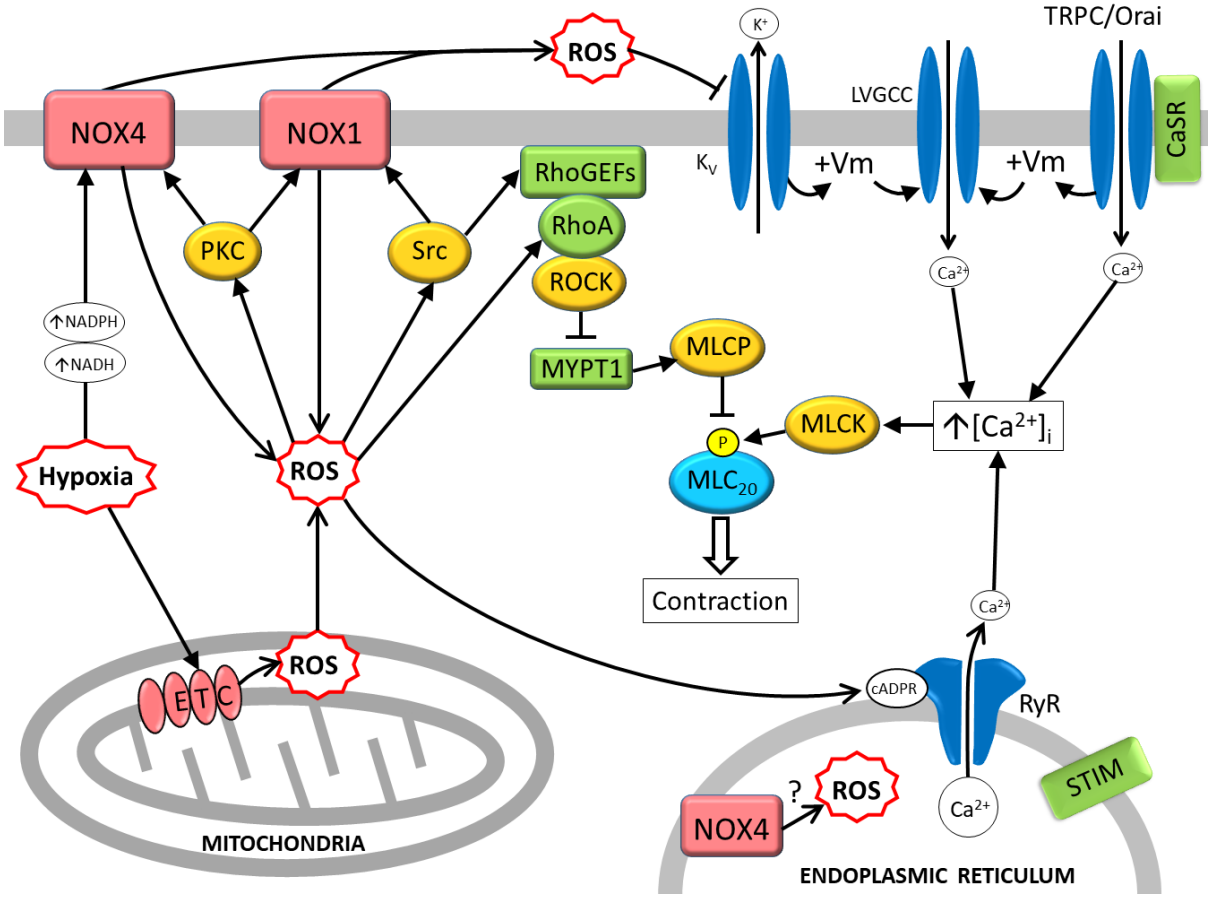

Figure 9. Proposed model for the role of NADPH oxidase (NOX) in hypoxic pulmonary vasoconstriction (HPV). Hypoxia may preferentially activate NOX4 by increasing the availability of cytosolic NADPH and NADH and/or by triggering an increased production of ROS from the mitochondrial electron transport chain (ETC) which activate PKC-ε and/or Src to phosphorylate regulatory subunits of NOX1 (PKC and Src) and directly phosphorylate NOX4 (PKC only). NOX-derived ROS may then raise $[Ca^{2+}]_i$ through a combination of K_V channel inhibition, TRPC/Orai store-operated Ca^{2+} channel opening and subsequent L-type voltage-gated Ca^{2+} channel opening (LVGCC). TRPC channel opening may be further stimulated via ROS-dependent activation of the Ca^{2+}-sensing receptor (CaSR). ROS also trigger Ca^{2+} release from the sarcoplasmic reticulum through ryanodine receptor channels (RyR) via enhanced production of cyclic ADP-ribose (cADPR). It is not known whether NOX4 in the sarcoplasmic reticulum contributes to this. Ca^{2+} activates myosin light-chain kinase (MLCK) to phosphorylate myosin light-chain-20 (MLC_{20}) and trigger contraction. In parallel to an increase in $[Ca^{2+}]_i$, ROS may also inhibit myosin light-chain phosphatase (MLCP) activity to further enhance MLC_{20} phosphorylation. This most likely occurs via Rho-kinase-dependent inhibitory phosphorylation of MYPT-1, and ROS most likely activate Rho-kinase via direct activation of RhoA or via Src-dependent phosphorylation of Rho guanine nucleotide exchange factors (RhoGEFs). Open arrowheads indicate movement or production of a substance. Closed arrowheads indicate an effect or response.

Figure 10

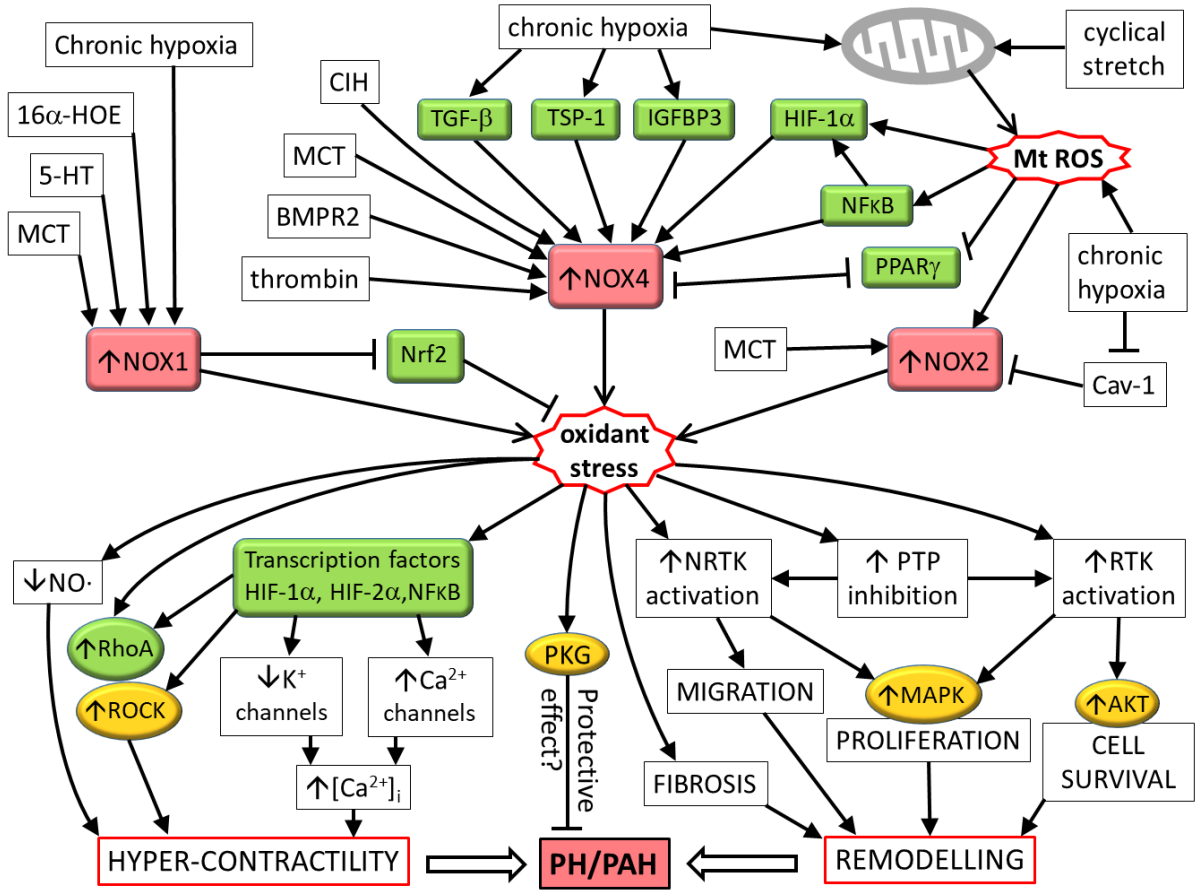
Chronic hypoxia
16α-HOE
5-HT
MCT
CIH
MCT
BMPR2
thrombin
chronic hypoxia
TGF-β
TSP-1
IGFBP3
HIF-1α
cyclical stretch
Mt ROS
NFκB
PPARγ
↑NOX4
chronic hypoxia
↑NOX1
Nrf2
MCT
↑NOX2
Cav-1
oxidant stress
↓NO·
Transcription factors HIF-1α, HIF-2α,NFκB
↑NRTK activation
↑ PTP inhibition
↑RTK activation
↑RhoA
↑ROCK
↓K+ channels
↑Ca2+ channels
↑[Ca2+]i
PKG
Protective effect?
MIGRATION
↑MAPK
PROLIFERATION
↑AKT
CELL SURVIVAL
FIBROSIS
HYPER-CONTRACTILITY
PH/PAH
REMODELLING

Figure 10. Proposed role of vascular smooth muscle NADPH oxidase (NOX) in the pathogenesis of pulmonary hypertension (PH) and/or pulmonary arterial hypertension (PAH). Multiple mitogenic and pro-contractile stimuli induce the increased expression of NOX1, NOX2 and/or NOX4. Chronic hypoxia and cyclical stretch act via mitochondrial ROS, the induction of HIF-1α and NFκB and suppression of PPARγ. Oxidant stress probably induces pulmonary arterial hyper-contractility through the combined effects of reduced NO$\cdot$ bioavailability, elevated $[Ca^{2+}]_i$, through suppression of K^+ channel expression and elevated Ca^{2+} channel expression, and enhanced RhoA/Rho-kinase expression and activity. Vascular remodelling occurs through the combined action of ROS-stimulated VSMC migration, proliferation, increased survival and fibrosis. These processes are promoted and co-ordinated by oxidative inhibition of protein tyrosine phosphatase (PTP) activity and enhanced activity of non-receptor tyrosine kinases (NRTK) and receptor tyrosine kinases (RTK). Counter to the pathogenic effects of oxidant stress, there may also be an underlying protective effect via activation of protein kinase G-1α (PKGIα) which may promote relaxation and suppress remodelling. MCT = monocrotaline, CIH = chronic intermittent hypoxia, 16α-HOE = 16α-hydroxyestrone, 5-HT = 5-hydroxytryptamine, BMPR2 = bone morphogenetic protein receptor-2, TSP-1 = thrombospondin-1, IGFBP3 = IGF binding protein-3, HIF-1α = hypoxia-inducible factor-1α, HIF-2α = hypoxia-inducible factor-2α, NFκB = nuclear factor kappa-light-chain-enhancer of activated B cells, PPARγ = peroxisome proliferator-activated receptor gamma, Nrf2 = nuclear factor erythroid 2-related factor 2, Cav-1 = caveolin-1, ROCK = Rho-kinase, Open arrowheads indicate movement or production of a substance. Closed arrowheads indicate an effect or response.

Table 1. Effects of vasoactive and mitogenic stimuli on ROS production in vascular tissues and cells. DHE = dihydroethidium, DCF = 2,7-dichlorofluorescein. Note that in many of the publications cited here DHE, LO-12 and Lucigenin are nominally used as selective probes for superoxide, while DCF and Amplex Red are nominally assumed to be selective for extracellular and intracellular H_2O_2 respectively. However, for some probes the accuracy and validity of this assumed selectivity may depend on the sample preparation and detection methods use [649]. VSMCs = vascular smooth muscle cells.

stimulus	Species and vascular bed	Indicator(s)	Effect(s)	reference
Intact Tissue				
Angiotensin II	Mouse aorta Mouse renal afferent arterioles Rat and mouse mesenteric artery Human internal mammary artery	Lucigenin DHE LO-12 cytochrome C reduction	Increased superoxide	[89, 94, 98, 101, 101, 104, 106]
	Rat cerebral artery Mouse renal afferent arterioles Rat renal afferent arterioles Human subcutaneous arteries	Tempo-9AC H2DCFDA/DCF	Increased H_2O_2	[82, 99, 105, 325, 377]
Prostanoids	Mouse aorta Mouse mesenteric artery Bovine coronary artery Rat pulmonary artery	L-012	Increased superoxide	[66, 140, 140, 174]
sphingosylphosphoryl choline	Mouse Mesenteric	C-DCFH/DA	Increased H_2O_2	[148]
Other GPCR agonists	Rat tail artery Human coronary artery Rat or mouse pulmonary artery Rat carotid artery	Lucigenin DHE	Increased superoxide	[113, 114, 135, 138, 178]
Perfusion pressure & stretch	Mouse afferent arterioles Rat femoral artery Bovine coronary artery Bovine pulmonary artery	DHE Lucigenin ethidium bromide	Increased superoxide	[162, 194, 334, 353]
	Mouse afferent arterioles	DCF	Increased H_2O_2	[230, 327]
Flow/ shear-stress	Mouse cerebral arteries (endothelium in situ) Human coronary arterioles (endothelium in situ)	DCF	Increased H_2O_2	[408, 418]
Flow overload Flow reversal	Porcine carotid artery	Electron paramagnetic resonance LO-12	Increased superoxide	[161, 422]
hypoxia	Mouse and porcine pulmonary artery	Cytochrome C reduction assay Lucigenin	Increased superoxide	[165, 650]
	Porcine, mouse and rat pulmonary artery	DCF	Increased H_2O_2	[588, 589, 650]
stimulus	**Species and vascular bed**	**indicator**	**effect**	**reference**
VSMCs				
Angiotensin II	Mouse and rat Aortic VSMC Rat renal afferent arteriole VSMCs Human resistance vessel VSMCs Bovine pulmonary artery VSMCs	DHE, Electron spin resonance spectroscopy Lucigenin Cytochrome C reduction	Increased superoxide	[72, 75, 80, 90, 95, 108, 133, 157, 163, 169]
	Rat aortic VSMCs Human aortic VSMC Human resistance vessel VSMCs Human coronary artery SMCs Human VSMC cell line	DCF Amplex red	Increased H_2O_2	[26, 45, 77, 79, 93, 96, 100, 102, 107, 170]
Prostanoids and eicosanoids	Bovine pulmonary artery SMCs Rat aortic VSMCs Mouse and bovine pulmonary VSMC	Cytochrome C reduction assay DHE lucigenin	Increased superoxide	[58, 142, 147-149]
	Rat aortic VSMCs Rat pulmonary VSMC	DCF	Increased H_2O_2	[145, 146, 651]

Other GPCR agonists	Rat and mouse aortic VSMCs Rat cerebral artery SMCs	Lucigenin DHE L012	Increased superoxide	[109, 117, 125, 126, 152]
	Rat and mouse aortic VSMCs Human coronary artery myocytes	DCF	Increased H_2O_2	[116, 121, 130, 136, 225]
Cytokines and growth-factors	Mouse, rat and human aortic VSMC	Lucigenin, DHE, LO-12 H2FF-BSA (endosomal specific) Electron spin resonance	Increased superoxide	[126, 163, 222, 229, 252, 255]
Cytokines and growth-factors	Mouse and rat aortic VSMC Human pulmonary VSMC	DCF Homovanillic acid assay FAT-HyPer (focal adhesion targeted H_2O_2 probe)	Increased H_2O_2	[130, 163, 187, 203, 210, 267]
stimulus	**Species and vascular bed**	**indicator**	**effect**	**reference**
Endothelium				
Angiotensin II	Mouse aortic ECs Human skin microvascular ECs Human coronary microvascular ECs	DHE Electron spin resonance lucigenin	Increased superoxide	[55, 72, 97]
Vasodilator agonists (acetylcholine, histamine, bradykinin)	Mouse mesenteric ECs Rat femoral artery ECs Human umbilical vein Cs Human and bovine aortic ECs Bovine pulmonary ECs Porcine aortic ECs	DCF	Increased H_2O_2	[237, 402, 405, 416, 417]
Other GPCR agonists	HMEC-1 (cell line) Human skin microvascular ECs	lucigenin DHE	Increased superoxide	[55, 124, 144, 232]
Laminar shear-stress	Rat basilar artery ECs Bovine aortic ECs	DHE Cytochrome C assay Electron spin resonance	Increased superoxide	[291, 419-421]
	Bovine aortic ECs	DCF, pHyper	Increased H_2O_2	[414]
Laminar shear-stress (prolonged)	Mouse aortic ECs	DHE, electron spin resonance	Decreased superoxide	[443]
oscillatory shear-stress or cessation of flow	Human umbilical vein ECs Mouse and bovine aortic ECs	DHE electron spin resonance	Increased superoxide	[414, 419, 431, 443, 652]
	Mouse lung capillary ECs Mouse pulmonary artery ECs Mouse and bovine aortic ECs	DCF Amplex red	Increased H_2O_2	[431, 432, 444]
Cytokines and growth factors	Mouse and human pulmonary ECs Human umbilical vein ECs	Fe^{2+} oxidation xylenol orange Amplex Red	Increased H_2O_2	[52, 158, 221, 223]

Table 2. Effects of antioxidants and NOX inhibition on vascular smooth muscle contraction responses to GPCR agonists, pressure and hypoxia and on relaxation responses to GPCR agonists and flow/shear stress. Note that drugs commonly used as non-selective NOX inhibitors, particularly diphenyleneiodonium (DPI) and apocynin are no longer considered selective enough for NOX and may indeed simply be acting as antioxidants [337], so any results with these drugs should be supported with the use of more selective drugs or specific NOX isoform siRNA or knockout. NaC = N-acetyl cysteine. SOD = superoxide dismutase.

Stimulus	Species and vascular bed	Inhibitor and effect	Target	Inferred role of Target	Reference
Pro-contractile GPCR agonists					
Angiotensin II-induced contraction	Rabbit aorta Rat cerebral Mouse mesenteric Mouse renal afferent arterioles Rat pulmonary artery	Tiron, Tempol, PEG-catalase, SOD-tg, apocynin DPI $p47^{phox-/-}$ NOX1 siRNA Gp91ds-tat All inhibit contraction	ROS NOX1 NOX2	ROS, NO1 and NOX2 = Pro-contractile	[89, 99, 104, 105, 133, 325, 335, 377, 378]
Phenylephrine-induced contraction	Mouse aorta Rat Tail artery	Tiron, NaC, apocynin, VAS2870 catalase, $Poldip2^{+/-}$ all inhibit contraction	ROS NOX4	ROS and NOX4 = Pro-contractile	[131, 135, 226]
ET-1-induced contraction	Rat pulmonary artery Mouse mesenteric artery	Tiron N-Acetyl cysteine Both inhibit contraction	ROS	ROS = Pro-contractile	[66, 114]
Sphingosine phosphorylcholine -induced Contraction	Rat mesenteric artery	Tempol, VAS2870 -inhibit $p47^{phox-/-}$ -inhibits but $gp91^{phox-/-}$ has no effect	ROS NOX1	NOX1 = Pro-contractile but NOX2 is not	[148]
U46619-induced contraction	Bovine coronary artery Mouse mesenteric artery Rat pulmonary artery	NaC Peg-catalase, Tempol, ebselen, apocynin, DPI gp91-ds-tat, $NOX2^{-/-}$, $p47^{phox-/-}$ All inhibit contraction	ROS NOX2	ROS and NOX2 = Pro-contractile	[66, 140, 146, 174]
Adenosine-induced contraction	Mouse aorta	Apocynin, PEG-SOD/Cat All inhibit contraction	ROS NOX	NOX = Pro-contractile	[116]
5-HT-induced contraction	pulmonary (mouse)	SOD, apocynin Both inhibit contraction but $gp91^{phox-/-}$ has no effect	Superoxide Not NOX2	Superoxide = Pro-contractile Not NOX2-derived	[138]
Stretch/pressure					
Myogenic contraction	Mouse renal afferent arterioles	PEG-SOD, tempol, ebselen, apocynin, $p47^{phox-/-}$ All inhibit contraction	superoxide NOX1 or 2	NOX1/2 = Pro-contractile	[99, 104, 230, 327, 334]
		$POLDIP2^{+/-}$. PEG-catalase Both inhibit contraction	NOX4, H_2O_2	NOX4-derived H_2O_2 = Pro-contractile	[230, 327]
Stretch induced contraction	Bovine coronary artery Bovine pulmonary artery	Apocynin, DPI, ebselen All inhibit contraction	ROS NOX	NOX = Pro-contractile	[194, 353]
Hypoxia					
Hypoxic pulmonary vasoconstriction	Mouse pulmonary artery	Apocynin, $p47^{-/-}$ P47 translocation inhibitor	NOX, ROS	NOX contributes to HPV	[165]
	Porcine and guinea-pig pulmonary artery	SOD inhibits DETCA (SOD inhibitor) potentiates	superoxide	Superoxide contributes to HPV	[650, 653]
	Mouse and bovine pulmonary artery	$gp91^{phox-/-}$ no effect $p47^{phox-/-}$ partial inhibition	NOX1	NOX1 but not NOX2 contributes to HPV	[578]
		NOX2 siRNA –no effect NOX4 siRNA –partial inhibition	NOX2 NOX4	NOX4 contributes to HPV bit NOX2 does not	[579]
Stimulus	**Species and vascular bed**	**Inhibitor and effect**	**Target**	**Inferred role of Target**	**Reference**

Pro-relaxant GPCR agonists					
Acetylcholine-induced relaxation	Rat aorta Mouse mesenteric artery Cat cerebral artery Dog coronary artery Rat femoral artery	SOD restores impaired relaxation Catalase, eNOS $^{-/-}$ inhibit relaxation	H_2O_2	H_2O_2 = Pro-relaxant (eNOS-derived) H_2O_2 = Pro-relaxant	[102, 400, 402, 405, 437, 462]
	Mouse mesenteric artery	NOX1$^{-/-}$ improved relaxation	NOX1, ROS	NOX1 = Pro-contractile	[424]
	Aorta from renovascular hypertensive mice	NOX2 $^{-/-}$, gp91ds-tat, Tiron, PEG-SOD All restore impaired relaxation	NOX2	NOX2 = pro-contractile	[425]
	Perfused cerebral artery from mouse	PEG-cat/gp91ds-tat Both inhibit relaxation	NOX2/ H_2O_2	NOX2 = Pro-relaxant	[418]
	Mouse aorta and perfused heart	Endothelium-specific NOX4 over-expression enhances relaxation and reduces perfusion pressure	NOX4, H_2O_2 (not superoxide)	NOX4 = Pro-relaxant	[215]
	Mouse mesenteric artery	Human NOX5 expression in VSM Inhibits relaxation	NOX5	NOX5 = Pro-contractile	[66]
Bradykinin-induced relaxation	Perfused mouse mesenteric artery Human mesenteric artery	SOD-/- inhibits increased flow)Catalase inhibits hyper-polarisation and relaxation	H_2O_2	H_2O_2 = Pro-relaxant	[404, 438]
Adenosine-induced relaxation	Rat cerebral artery	PEG-SOD, PEG-Cat	ROS	ROS = pro-relaxant	[117]
Histamine-induced relaxation	Mouse aorta and perfused heart	EC-specific NOX4 over-expression enhances relaxation response and reduces perfusion pressure	H_2O_2	NOX4 = Pro-relaxant H_2O_2 = Pro-relaxant	[215]
Flow/ shear-stress					
Flow/ shear-stress Induced relaxation	Human coronary artery Rat mesenteric artery Human coronary arterioles Mouse cerebral arteries	Catalase, PEG-catalase gp91ds-tat All inhibit relaxation	H_2O_2	H_2O_2 = pro-relaxant NOX2 = pro-relaxant	[408, 410, 411, 418]

Table 3. Effects of exogenous reactive oxygen species (ROS) on vascular tone. Pro-contractile and pro-relaxant effects of H_2O_2 or superoxide (generated extracellularly with xanthine/xanthine oxidase (X/XO) or intracellularly with LY83583) on arterial preparations *in vitro* or blood flow *in vivo*.

Pro-contractile effects of ROS					
ROS stimulus	**Pre-constriction?**	**Vascular bed**	**Bath conc.**	**effect**	**reference**
Superoxide (X/XO, LY83583)	None	Rat aorta and pulmonary artery	1-400μM	Causes contraction	[311, 339, 340]
	$PGF_{2\alpha}$ U46619/L-NAME	Rat pulmonary artery, mesenteric artery and femoral artery	1-10μM	Enhances pre-constriction	[174, 311, 351]
	Pressure/myogenic	Mouse renal afferent arteriole Rat cerebral artery	0.1-250μM	Enhances pre-constriction	[325, 327]
	KCl	Rat pulmonary and ,mesenteric artery	1-10μM	Enhances pre-constriction	[148, 351]
	Permeabilised, pCa 6.9	Rat pulmonary artery	10μM	Enhances pre-constriction	[311]
H_2O_2	None	Rat and mouse aorta Rat mesenteric artery Bovine coronary artery Canine cerebral artery SHR carotid artery Mouse carotid artery Rat, rabbit & porcine pulmonary artery	1μM – 10mM	Causes contraction	[131, 146, 194, 283, 326, 340-342, 342, 343, 343-348, 515]
	phenylephrine	Rat mesenteric artery	10-100μM	Enhances pre-constriction	[350]
	Pressure/myogenic	Rat mesenteric artery	100μM	Enhances pre-constriction	[343]
	KCl	Mouse aorta Mouse mesenteric artery Mouse carotid artery Bovine pulmonary artery	1μM-1mM	Enhances pre-constriction	[341, 341, 342, 342, 342, 352-354]
Pro-Relaxant Effects of ROS					
ROS stimulus	**Pre-constriction?**	**Vascular bed**	**Bath conc.**	**effect**	**reference**
Superoxide (LY83583, menadione, X/XO)	PGF2α U46619/L-NAME Phenylephrine	Rat mesenteric and femoral artery Rat aorta	10-50μM	Causes relaxation	[311, 333, 351]
	Myogenic	Cerebral circulation *in vivo* (rat)	200μM	Suppresses myogenic auto-regulation	[396]
H_2O_2	U46619 PGF2α ET–1 Noradrenaline Phenylephrine	Mouse, rat and human mesenteric artery Porcine coronary artery Human coronary arteriole Rat femoral artery Rabbit iliac artery	1μM-10mM	Causes relaxation	[336, 350, 354, 400-407]
	Pressure	Coronary circulation *in vivo*	1-300μM	Increases blood flow	[336]
	Myogenic	Mouse renal afferent arteriole Human coronary arterioles Mouse mesenteric artery Rat coronary artery	1μM-1mM 25μM	Causes relaxation	[327, 334, 354, 408-411]
	Hypoxia	Guinea-pig pulmonary artery	10-300μM	Causes relaxation	[653]

Table 4. Effects of antioxidants and NOX inhibition on VSM growth/proliferative/migration & associated signalling in response to mitogenic and hypertensive stimuli. DPI = diphenyleneiodonium. NaC = N-acetyl cysteine. SOD = superoxide dismutase.

stimulus	Species and vascular bed	Inhibitor and effect	Target	Inferred role of target	reference
Proliferation					
Angiotensin II induced proliferation	Mouse and rat aortic VSMC Human resistance artery VSMCs	Apocynin, DPI $P22^{phox}$ siRNA NOX1 siRNA NOX1-/- NOX2 siRNA All inhibit proliferation	NOX1/2	NOX1 and NOX2 = pro-proliferative	[79, 107, 490, 560]
	Aorta (mouse)	NOX4 over-expression Does NOT influence proliferation	NOX4/ H_2O_2	NOX4 is not pro-proliferative	[215]
Other GPCR agonists (Thrombin, ATP, Urotensin II, 5-HT) Induce proliferation	Rat and human aortic VSMC Rat pulmonary VSMC	NaC DPI, apocynin NOXA1 shRNA NOX1-/- NOX4 siRNA All inhibit proliferation	NOX NOXA1	NOXA1 = pro-proliferative NOX1 = pro-proliferative NOX4 = pro-proliferative	[118, 121, 126, 127, 597, 645]
Growth factors (PDGF, TGF-β) Induce proliferation	Rat and human aortic VSMCs Human pulmonary VSMC	NaC, catalase NOX4 siRNA NOX5 siRNA All inhibit proliferation	ROS NOX5	NOX4 = pro-proliferative NOX5 = pro-proliferative	[187, 210, 267, 317]
Chronic hypoxia Induced VSMC proliferation and/or vascular remodelling	Mouse, human Pulmonary vasculature or VSMCs	NOX1-/- NOX2-/- NOX4 siRNA GKT137831 All inhibit pulmonary media thickening or induce apoptosis	NOX1 NOX2 NOX4	NOX1, NOX2 and NOX4 promote pulmonary vascular remodelling and PH	[59, 592, 599, 635, 644] [639]
	Mouse pulmonary vasculature	NOX4-/- No effect on pulmonary vascular remodelling	NOX4	NOX4 NOT involved in vascular remodelling in PH	[59, 647]
Monocrotaline induced VSMC proliferation	Rat pulmonary VSMC	NOX1 siRNA VCC588646 and VCC202273 All inhibit proliferation	NOX1 NOX4	NOX1 and NOX4 promote VSMC proliferation and remodelling	[593, 595]
Other models of PAH Induced VSMC proliferation	Human pulmonary VSMC Lamb pulmonary VSMC Rat pulmonary VSMC	NOX4 siRNA GKT137831 Inhibit proliferation	NOX4	NOX4 = pro-proliferative	[59, 210, 629, 642]
RTK transactivation and ERK phosphorylation					
Angiotensin II	Rat aortic VSMCs	Nac, Tiron, α-tocopherol, DPI $P22^{phox}$ siRNA All inhibit EGFR transactivation and ERK phosphorylation	NOX, ROS	NOX = pro-proliferative	[90, 95, 103, 274, 560]
Other GPCR agonists ET-1, thrombin, phenylephrine, Lyso-phosphatidyl choline, Sphingosine 1-phosphate, urotensin II Induced EGFR transactivation and/or ERK phosphorylation	Rat and mouse aortic VSMCs	NAC, Tiron catalase, DPI, apocynin dom neg p47 NOX1-/- and NOX1 siRNA All inhibit EGFR transactivation and ERK phosphorylation	ROS NOX1	NOX1 = pro-proliferative	[64, 109, 110, 121, 125, 130, 136, 147, 149, 156]
Growth factors (PDGF, TGF-β) Induce ERK phosphorylation	Rat aortic VSMCs Human pulmonary VSMC	Catalase or NaC NOX4 siRNA Inhibit ERK phosphorylation	NOX4	NOX4 = pro-proliferative	[210, 267]
stretch	Bovine coronary artery	Ebselen, DPI, apocynin Inhibit EGFR transactivation and ERK phosphorylation	ROS/NOX	NOX = pro-proliferative	[194]
VSM Hypertrophy					
Angiotensin II Induced VSMC hypertrophy	Rat and mouse aortic VSMCs	NaC, DPI, $p22^{phox}$ antisense NOX1 siRNA All inhibit hypertrophy	NOX1, 2 or 4	NOX1/2/4 promote growth	[89, 96, 157, 169]

		$p22^{phox}$ overexpression in VSMC Enhances hypertrophy	ROS/NOX1/2/4	NOX1/2/4 promote growth	[552]
PI3K/AKT signalling and protein synthesis					
Angiotensin II	Human resistance artery VSMCs Rat aortic VSMCs	DPI NOX1 siRNA Inhibit AKT phosphorylation and protein synthesis	NOX1	NOX1 promotes growth	[79, 107]
Other GPCR agonists ET-1, thrombin, phenylephrine, Sphingosine 1-phosphate	Rat aortic VSMCs	NAC, ebselen, Apocynin, DPI NOXA1 shRNA Inhibit PI3K activation, AKT activation and protein synthesis	NOX1	NOX1 and NOXA1 promote growth	[109, 126, 136, 150]
VSMC Migration					
Angiotensin II Induced VSMC migration	Human coronary artery SMCs Rat aortic VSMCs	NAC, DPI $P22^{phox}$ siRNA NOX1 siRNA Inhibit migration	NOX1	NOX1 stimulates migration	[100, 107, 560]
Other GPCR agonists (ET-1, thrombin, Sphingosine 1-phosphate, urokinase) Induced VSMC migration	Rat aortic VSMCs Human coronary artery	DPI, apocynin NOX1-/- (inhibits migration)	NOX1	NOX1 stimulates migration	[125, 147, 178]
Growth factors (PDGF, IGF, TNF-α) Induce VSMC migration	Rat and mouse aortic VSMCs	Catalase, NAC NOX1-/- NOX4 siRNA, POLDIP2 siRNA (inhibits focal adhesion turnover and migration)	NOX1 NOX4	NOX1 and NOX4 stimulate migration	[163, 222, 229, 267]

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